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Bridging the translational gap between rodent and human pain research

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Bridging the Translational Gap between Rodent and Human Pain Research
by

Tayler Diane Sheahan

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Tayler Sheahan, Washington University in St. Louis, December 2017

ABSTRACT OF THE DISSERTATION

Bridging the translational gap between rodent and human pain research

by

Tayler Diane Sheahan

Doctor of Philosophy in Biology and Biomedical Sciences

Neurosciences

Washington University in St. Louis, December 2017

Professor Robert W. Gereau IV, Chair

The treatment of chronic pain is an immense clinical and societal burden rooted in the ineffectiveness and adverse side effects of existing analgesics. Extensive efforts have been directed towards the development of novel pain therapies with maximal efficacy and minimal unwanted effects; however, putative therapeutic targets identified in preclinical rodent models rarely translate in clinical trials. The poor translational record of basic pain research findings has been attributed, in part, to the use of suboptimal rodent pain models and behavioral endpoints used to assess putative analgesics, as well as differences in the pharmacological profiles of rodents and humans. The work presented in this thesis aims to address these limitations.

Human pain is defined as a complex sensory and emotional experience, yet rodent pain models have historically used reflex/withdrawal measures of hypersensitivity as the primary outcome. To address this limitation, the first study of this thesis evaluates more complex, voluntary behaviors as indicators of pain-like behavior in rodents. We found that inflammation and nerve injury minimally interfere with physical activity (voluntary wheel running, locomotion, and gait), social interaction, or anxiety-like behavior in mice, indicating that these voluntary behaviors are not reliable pain-related readouts across rodent injury models. As recent findings from other groups align with our results, we further conclude that in contrast to humans, changes in these voluntary behaviors are not characteristic of persistent pain in mice.

Although rodents and humans possess different pharmacological profiles, putative analgesics are oftentimes identified and exclusively evaluated in rodent tissues and/or pain models prior to entering clinical trials. In response to this translational gap, we recently developed a protocol to surgically extract dorsal root ganglia from deceased human organ donors and subsequently culture sensory neurons. In the second study of this thesis, we utilize human sensory neurons to assess the translational potential of targeting metabotropic glutamate receptors 2 and 3 (mGluR2/3), which have been identified as modulators of pain in a variety of rodent models. In mouse sensory neurons, we found that activation of mGluR2/3 blocked inflammation-induced sensitization of the nonselective cation channel TRPV1. In contrast, this effect was not observed in human sensory neurons. These results indicate that mechanisms of peripheral analgesia are not entirely conserved across species. More broadly, our findings demonstrate that using human tissue to validate analgesic targets identified in rodents is an important step in the translational research process.

Due to poor pain relief from current pharmacological therapies, exercise has been explored as an alternative, nonpharmacological intervention for chronic pain. Indeed, exercise has been shown to improve patient pain ratings and functionality, albeit via largely unknown mechanisms. In the final study of this thesis, we evaluated whether voluntary exercise similarly reduced pain-like behavior in mice, with the goal of using a mouse model to elucidate the molecular mechanisms mediating clinical exercise-induced analgesia. However, we found that voluntary wheel running did not reduce pain-like behavior in common rodent models of inflammation and nerve injury. Previous preclinical studies of exercise-induced analgesia utilized forced exercise paradigms, and thus our findings suggest that voluntary and forced exercise may have different analgesic potential in rodents.

Taken together, there are a variety of existing experimental limitations that can be addressed to increase the translatability of basic pain research. Based on our current findings, we conclude that voluntary rodent behavioral endpoints modeled off of the human chronic pain

experience have limited utility. In contrast, confirming preclinical findings in human tissue represents a promising approach to bridge the translational gap between rodent and human pain research.

Chapter 1

Introduction to pain and experimental approaches in basic pain research

1.1 Chronic pain is a public health priority

The treatment of chronic pain has been an immense societal and clinical burden for centuries.¹³³ Recent estimates indicate that in the United States alone, chronic pain afflicts over 100 million people and costs \$635 billion annually with respect to health care expenses and productivity loss.^{60,75} These figures are likely to increase given the current aging population.^{67,125} Common chronic pain conditions include low back pain, arthritis, migraine, and fibromyalgia. Further, chronic pain is often associated with diabetes, cancer, and chemotherapy.^{53,75,151} Notably, these chronic pain conditions and others are frequently accompanied by physical disability and mood disorders, which further impair patient quality of life.^{49,64,76,120,156} Chronic pain is a nondiscriminatory disease: it affects populations irrespective of age, sex, ethnicity, and socioeconomic status (SES). However, higher incidence and severity of pain have been reported in women, older adults, and those of lower SES.^{15,53,61,125,151} Thus, given its pervasiveness and societal impact, chronic pain is gaining prominence as a public health priority.^{15,61}

Treating chronic pain has been an ongoing problem rooted in the ineffectiveness and/or adverse side effects of existing analgesics including opioids and nonsteroidal anti-inflammatory drugs (NSAIDs).^{17,54,55,75,143} Although opioids provide robust analgesia in some cases, the recent rise of opioid prescriptions for the treatment of chronic pain has manifested in an alarming increase in opioid addiction and misuse, which has become an epidemic in its own right.^{82,90} Therefore, it is pressing that we develop new, efficacious therapies that could bypass the centrally mediated side effects of existing analgesics. In response to the poor pain relief from pharmacological interventions, exercise, mindfulness meditation, and pain management therapy have been explored as nonpharmacological interventions and have been shown to improve pain ratings and functionality in many cases, albeit via largely unknown mechanisms.^{38,58,170}

However, as nonpharmacological/lifestyle interventions cannot be implemented by all chronic pain patients, new drug therapies are desperately needed.

Over the past 50 years, substantial efforts have been directed towards the development of novel analgesics, yet there has been little improvement in clinical pain treatment options.^{55,86,116} Upon evaluation of years of repeated analgesic failure, the poor translational record of basic pain research findings has been attributed to a combination of limitations including 1) flawed clinical trial design and implementation, 2) differences in drug pharmacological profiles between rodents and human, and 3) the use of suboptimal rodent pain models and behavioral endpoints in assessing putative analgesics.^{101,116,158} Therefore, the development of efficacious chronic pain treatments will require an enhanced understanding of the mechanisms underlying pain processing, as well as improved pain research methodologies.

1.2 Neurobiology of pain processing

Pain is defined as an unpleasant sensory and emotional experience associated with actual tissue damage, or described in terms of such damage.⁹⁷ Pain is an important survival mechanism that warns an individual of potential bodily danger. Under normal physiological conditions, following injury or tissue damage, pain serves to protect the affected area from further damage until the injury has resolved. For example, tibial stress fracture is a common injury faced by long-distance runners that results from repeated microtrauma to the bone and produces tenderness of the leg.^{52,102} While injured, a runner is likely to experience a shift in pain response intensities such that a stimulus or activity that was not painful previously, such as walking, becomes perceived as painful, which is called allodynia.^{7,97} Further, stimuli or activities that are typically painful, such as applying pressure to the affected area, are perceived as exceedingly painful, which is referred to hyperalgesia.^{7,97} This pain serves as a warning mechanism to halt running until the injury has healed. In contrast, peroneal nerve entrapment

elicited by running causes prolonged pain that can continue even after surgical treatment.⁹⁴ Pain that persists despite recovery from an injury (or in the absence of any injury) and no longer serves as a protective mechanism is referred to as chronic pain.

The nervous system detects and processes noxious stimuli in a process called nociception (Fig. 1).⁹⁷ Specialized sensory neurons of the peripheral nervous system referred to as nociceptors detect damaging stimuli via free nerve endings that innervate the skin and viscera.¹⁶¹ Noxious thermal, chemical, and mechanical stimuli act on high-threshold ion channels to cause nociceptor membrane potential depolarization.⁶⁸ Sufficient depolarization elicits an action potential at the peripheral nerve terminal that is propagated along the axon, through the cell body located in the dorsal root ganglia (DRG), and to the central nerve terminals in the spinal cord dorsal horn (SCDH). In the SCDH, action potential propagation to central nerve terminals culminates in synaptic release of neurotransmitters and neuropeptides onto secondary SCDH neurons of the central nervous system.^{16,161}

Notably, in addition to the SCDH, nociceptive inputs originating at peripheral nerve terminals can also be transmitted back to the periphery (a phenomenon referred to as axon reflex⁴⁸) or to the spinal cord ventral horn to mediate nociceptive withdrawal reflexes. Axon reflex arises when an action potential propagates locally along multiple peripheral branches of a single nociceptive afferent and causes the peripheral nerve terminals to release glutamate and peptides such as substance P and calcitonin-gene related peptide (CGRP).^{43,123,160} These events can drive neurogenic inflammation by promoting immune cell activation and vasodilation.^{66,99} Nociceptive withdrawal reflexes result when nociceptive inputs activate spinal interneurons that directly synapse on motor neurons within the spinal cord ventral horn.¹³⁶ Motor neuron activation elicits a rapid withdrawal response from the painful stimulus, providing protection from further tissue damage, as when one places her hand on a hot stove.

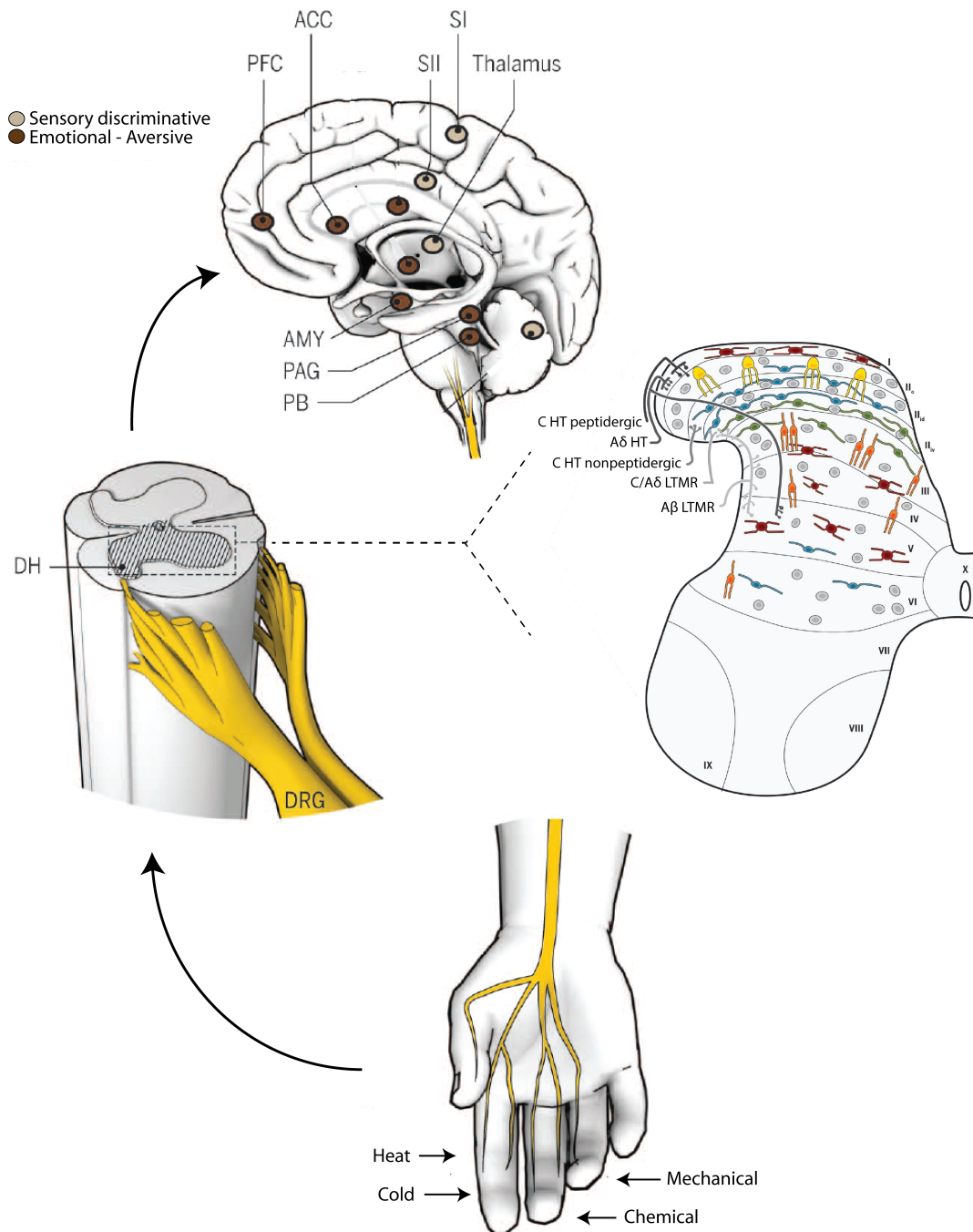


Figure 1. Nociceptive and somatosensory circuits. A variety of stimuli can activate cutaneous fibers of dorsal root ganglia (DRG) neurons that project to the dorsal horn (DH) of the spinal cord. Within the DH, the central terminals of C and Aδ high threshold nociceptors (HT) and low-threshold mechanoreceptors (LTMR) project to distinct lamina and synapse on a variety of neuronal populations. Noxious inputs mediated by HT primary afferents terminate in superficial lamina I through the dorsal portion of inner lamina II (II_{id}) while innocuous inputs mediated by LTMRs terminate in the ventral portion of inner lamina II (II_{iv}) through lamina V. From the DH, inputs are relayed by ascending projection neurons to the thalamus and brainstem and then onto brain regions that mediate the sensory discriminative (light brown circles) and emotional-affective (dark brown circles) of pain. PFC, prefrontal cortex; ACC, anterior cingulate cortex; SI/SII, primary/secondary somatosensory cortex; AMY, amygdala; PAG, periaqueductal gray region, PB, parabrachial nucleus. Adapted from Peirs et al. 2016 with written permission from Rebecca Seal.¹²⁶

From the SCDH, ascending projection neurons relay nociceptive inputs to the thalamus and brainstem, which further disseminate inputs to a variety of brain regions. To elaborate, thalamic nuclei extend projections to the somatosensory cortices, which encode nociceptive stimulus location, duration, and intensity. Thalamic nuclei also extend projections to the anterior cingulate and prefrontal cortices, which underlie emotional responses to noxious stimuli.^{2,7,16} Projections from the parabrachial nucleus within the brainstem to the hypothalamus and amygdala mediate autonomic and affective responses to nociception, respectively.² Additional inputs to brainstem nuclei such as the rostral ventral medulla and periaqueductal gray engage descending pathways that modulate nociceptive inputs at the spinal cord level.¹⁶ Together, activation of these brain regions and others – collectively termed the pain neuraxis – yields the complex, aversive sensory and emotional perception of pain. Dysregulation of nociceptive processing at any level of the pain neuraxis can produce chronic pain.⁶⁸

1.3 Molecular determinants of nociceptor functional diversity

1.3.1 Sensory neurons are functionally diverse

That we can perceive the nature of an acute noxious stimulus – that is hot, cold, mechanical, or chemical – has long intrigued scientists and physicians.¹³⁸ After over a century of research, we now know that our ability to detect a wide range of noxious stimuli arises from the diversity of primary sensory afferents, which are classified based on axon diameter, extent of myelination, and conduction properties.¹³² A β afferents are large diameter, myelinated fibers with rapid conduction velocities that are broadly divided into two categories: low-threshold mechanoreceptors (LTMR) that convey innocuous mechanical inputs such as light touch and proprioceptors that encode body position and balance.¹ A δ afferents are medium diameter, thinly myelinated fibers that function as either LTMR or high-threshold (HT) nociceptors that carry noxious mechanical and/or thermal inputs, which are perceived as “fast pain”.¹³² The

majority of sensory neurons are slowly conducting C fiber afferents that are unmyelinated and small diameter.¹⁶¹ For the most part, C afferents are HT polymodal fibers that mediate “slow pain” in response to noxious thermal, chemical, and mechanical stimuli; however, C-LTMRs have also been identified in hairy skin.^{1,132,161} C fiber nociceptors can be further classified based on whether or not they express the neuropeptides CGRP and substance P, in which case they are termed peptidergic or nonpeptidergic C fibers, respectively. It has been suggested that these C fiber nociceptor subtypes are functionally distinct, with peptidergic nociceptors mediating responses to noxious heat, and nonpeptidergic nociceptors responding to noxious mechanical stimuli.^{27,103} In addition to these functional distinctions, the central terminals of primary afferent subtypes are somatotopically organized within the SCDH (Fig. 1). Neuroanatomical studies have revealed that A δ and C fibers synapse in the superficial lamina I, outer lamina II, and the dorsal part of inner lamina II, whereas A β fibers synapse deeper in the ventral part of inner lamina II through lamina V^{18,174}. In turn, these projections synapse onto unique subsets of secondary spinal cord neurons.¹²⁶

1.3.2 Transient receptor potential channels are the primary transducers of noxious stimuli

The functional identity of nociceptors is governed by the expression of receptors and ion channels at the peripheral nerve terminal. Although there is a large cast of molecular mediators that contribute to nociception, the leading roles are played by sensory transient receptor potential (TRP) channels. TRP channels are a family of six-transmembrane domain, non-selective cation channels with high Ca²⁺ permeability that are activated by a variety of exogenous and endogenous stimuli such as temperature, plant-derived irritants, toxins, acidity, and bioactive lipids.^{80,109}

TRPV1, TRPM8, and TRPA1 have emerged as the primary transducers of noxious stimuli. Briefly, TRPV1, formerly known as vanilloid receptor 1, is activated by temperatures >

42°C, chemical ligands including the pungent ingredient from spicy chili peppers called capsaicin, low extracellular pH, and bioactive lipids.^{26,106} The functional significance of TRPV1 in nociception was first demonstrated by impaired behavioral responses to noxious heat in mice lacking the receptor.²⁵ Since this seminal discovery, TRPV1 has also been established as a mediator of inflammation-induced peripheral sensitization and neuropathic pain (further discussed below).^{8,25,41,81} The chief cold-sensitive ion channel within sensory neurons is TRPM8, which responds to cooling temperatures roughly < 25°C, as well as menthol and eucalyptol.¹⁰⁵ Analogous to TRPV1, mice lacking TRPM8 display decreased sensitivity to environmental cold.^{11,35,45} TRPA1, sensitive to garlic, cinnamon, and mustard oil, has also been proposed to mediate responses to cold temperatures < 15°C^{4,144}; however, its role as an *in vivo* cold-sensor has been fiercely debated.^{92,104} While some studies of TRPA1-deficient mice show attenuated behavioral responses to noxious cold^{83,91}, others find no effect of eliminating TRPA1.^{9,87,169} Despite this controversy, sensory neuron TRPA1 is widely accepted as an important chemoreceptor for environmental irritants^{9,121}, and has also been suggested to mediate and/or modulate mechanosensation.^{91,169} This finding is of particular interest, as the identity of the primary mammalian high-threshold mechanoreceptor remains an enigma.^{10,73,157} Other candidate mechanotransducers have been suggested, including Piezo proteins^{85,157}, acid sensitive ion channels¹⁴², and TRPV4⁵⁷, yet further investigation regarding nociceptive mechanoreceptors is required.

Notably, genetic knockout of TRPV1 or TRPM8 impairs, but does not fully abolish sensitivity to noxious heat or cold stimuli, respectively.^{11,25,35,41} Thus, functional redundancy of ion channels likely facilitates modality-specific detection of potentially harmful stimuli.⁷ Nevertheless, much of our current understanding regarding the peripheral somatosensory processing of distinct modalities has resulted from studying sensory neurons expressing TRPV1 or TRPM8, which are found in largely non-overlapping populations under uninjured conditions.⁸⁸

1.4 Modulation of nociceptive input and peripheral sensitization

Membrane-bound G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) play a critical role in the modulation of nociceptive inputs.^{7,28,161} Following injury, damaged neuronal and nonneuronal cells such as keratinocytes and infiltrating immune cells release a wide array of signaling molecules into the extracellular environment that comprise the “inflammatory soup”, including growth factors (nerve growth factor), peptides (CGRP, substance P, bradykinin), glutamate, prostaglandins, chemokines, and many others.¹³² These inflammatory mediators can directly influence sensory neurons by binding to their respective GPCR or RTK on peripheral nerve terminals, which activate multiple intracellular signaling cascades that have diverse downstream effects on nociceptors including 1) enhanced activity of ion channels and receptors that transduce noxious stimuli, 2) increased trafficking of nociceptive ion channels and receptors to the plasma membrane, 3) increased transcription and/or translation of nociceptive ion channels and receptors.^{7,109,161} These events culminate in increased responsiveness and decreased threshold of nociceptors, a form of neuronal plasticity termed peripheral sensitization.⁹⁷

Alterations in gene and protein expression caused by tissue insult and subsequent peripheral inflammation culminate in two important functional changes of injured nerve fibers and neighboring, intact fibers: phenotypic switching and ectopic activity.^{44,161} A β LTMR fibers undergo a phenotypic switch in which they express neuropeptides such as substance P *de novo*. In turn, A β LTMR fibers begin to convey both innocuous and noxious mechanical inputs, thereby expanding the proportion of primary afferents that convey nociceptive inputs to the SCDH.^{20,164} Behaviorally, A β phenotypic switching underlies tactile allodynia, as demonstrated by the rapid pain sensation experienced upon touching a cut or bruise, which could not be mediated by slow conducting C fibers.^{20,44} Further, “silent” C and A δ fiber cutaneous nociceptor afferents insensitive to mechanical stimuli under physiological conditions gain mechanical

sensitivity after exposure to inflammatory mediators.^{108,140,163} In addition to phenotypic switching, membrane hyperexcitability due to increased expression of voltage-gated sodium channels and decreased levels of voltage-gated potassium channels causes A β , C, and A δ sensory neurons to fire spontaneous action potentials as soon as hours or days after nerve injury.^{44,50,96,135,146} Ectopic activity within intact fibers that come in contact with injured fibers is believed to mediate spontaneous pain sensations.^{135,162} Taken together, peripheral sensitization broadens the repertoire of nociceptive inputs. Notably, these changes trigger and maintain central sensitization, thereby fostering the transition from acute to chronic pain.^{63,110,135} For this reason, blocking peripheral sensitization, with the idea of stopping pain at the source, remains an appealing therapeutic strategy. In fact, common drugs for pain relief such as NSAIDs work by reducing the production of proinflammatory and proalgesic mediators.⁷ Due to the plethora of extracellular inflammatory mediators, however, one might argue that directly reducing nociceptor sensitization rather than targeting a given inflammatory mediator may be a more effective way to reduce peripheral sensitization.

1.5 TRPV1 is a key mediator of peripheral sensitization

Soon after the prominent role of TRPV1 in nociception was discovered, pain researchers found that TRPV1 similarly played a vital role in peripheral sensitization. For example, mice lacking the TRPV1 receptor fail to develop inflammation-induced heat hypersensitivity.^{25,41} Likewise, pharmacological inhibition of TRPV1 can attenuate hypersensitivity to heat and/or mechanical stimuli in rodent models of nerve injury, diabetic neuropathy, and chemotherapy-induced neuropathy.^{8,81,95,152} These behavioral observations spurred interest in TRPV1 as a putative peripheral analgesic target and drove investigation of the molecular mechanisms underlying TRPV1 modulation under physiological and pathological conditions.

The influx of calcium at nociceptor peripheral nerve terminals following TRPV1 activation has widespread intracellular effects. Sufficient plasma membrane depolarization can activate voltage-gated sodium channels and drive the firing of action potentials. Further, increased intracellular Ca^{2+} engages a variety of intracellular signaling cascades that influence ion channel function. Under physiological conditions, TRPV1 undergoes Ca^{2+} -dependent desensitization upon dephosphorylation by calcineurin, a Ca^{2+} /calmodulin-dependent phosphatase.¹⁵⁰ Conversely, Ca^{2+} /calmodulin-dependent protein kinase II opposes Ca^{2+} -dependent desensitization by maintaining phosphorylation of TRPV1.¹⁵⁰ Thus, increased intracellular Ca^{2+} can bidirectionally modulate TRPV1 function.

Following tissue damage, TRPV1 is a prominent target of inflammatory mediators. As described above, inflammatory mediators influence ion channels at multiple levels, and TRPV1 is no exception. Foremost, proinflammatory mediators sensitize TRPV1 by reducing the activation threshold and potentiating the response magnitude to suprathreshold stimuli.^{7,8,80,109} This occurs either by direct ligand (i.e. endovanilloids and protons) binding to TRPV1 at positive allosteric sites or indirect modulation of TRPV1 via intracellular second messenger signaling cascades.^{7,8,80,109} For instance, the proinflammatory mediator prostaglandin E₂ (PGE₂) binds to the PGE G_s-coupled GPCR subtypes EP₃C and EP₄, stimulating adenylyl cyclase and the cAMP/PKA pathway (Fig. 2).^{107,141} Subsequent PKA phosphorylation of TRPV1 increases channel conductance and decreases channel

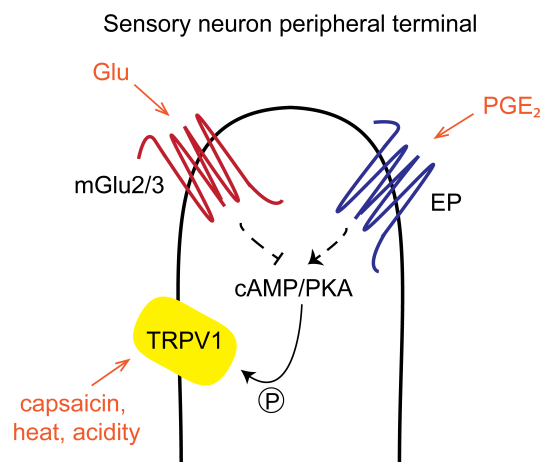


Figure 2. TRPV1 expressed on sensory neuron peripheral nerve terminals is sensitized by inflammatory mediators. Upon binding their receptor, inflammatory mediators such as PGE₂ stimulate the cAMP/PKA pathway, leading to TRPV1 phosphorylation (P) and sensitization. In rodents, activation of the G_i-coupled GPCRs mGlu2/3 suppresses TRPV1 sensitization by inhibiting the cAMP/PKA pathway and decreases nociceptor hyperexcitability.

desensitization.^{12,72,98,114,150} In addition to sensitization of TRPV1 channels already present in the plasma membrane, inflammatory mediators increase surface membrane expression of TRPV1. Nerve growth factor (NGF) has been shown to enhance membrane trafficking by inducing TRPV1 phosphorylation via Src family tyrosine kinases.^{78,109,171,172} Lastly, inflammation and nerve injury increase TRPV1 expression in sensory neurons. Retrograde transport of NGF from peripheral nerve terminals to DRG cell bodies increases TRPV1 translation.^{77,81} Notably, both enhanced TRPV1 protein in existing TRPV1⁺ neurons, as well as ectopic expression of TRPV1 receptors in previously TRPV1⁻, myelinated afferents has been observed post-injury – another example of primary afferent phenotypic switching following peripheral sensitization.^{71,129}

The prevalent role of TRPV1 in peripheral sensitization rendered it a prime pharmacological target for pain relief. Over-the-counter, low-concentration capsaicin creams are marketed for pain relief, although the ability of these creams to cause analgesia compared to placebo has been contested.¹⁴⁷ TRPV1 antagonists have also been widely explored in Phase I and II clinical trials; however, their clinical use has been limited largely due to hyperthermia resulting from activity at centrally expressed TRPV1 receptors.^{56,147} In cases of severe cancer pain, site-specific activation of TRPV1 by the extra-pungent capsaicin analog resiniferatoxin, which selectively causes excitotoxicity and ablation of TRPV1⁺ nociceptors while sparing other sensory afferents, has shown promise in both preclinical and clinical studies.^{19,69,100,147} However, resiniferatoxin is not a viable therapeutic strategy for all pain conditions. Although targeting TRPV1 for pain relief has been problematic, due to the presence of TRPV1 at peripheral nerve terminals and its essential role in peripheral sensitization, developing alternative strategies to modulate TRPV1 activity remains an enticing therapeutic strategy. Indeed, manipulating glutamate receptor intracellular signaling, which transmits and modulates nociceptive inputs, represents one such alternative strategy.

1.6 Glutamate transmits and modulates nociceptive inputs

Glutamate is the major excitatory neurotransmitter of the vertebrate nervous system and thus is pivotal to countless physiological and pathological processes including nociception, pain perception, and chronic pain. Glutamate activates two categories of receptors that are expressed throughout the pain neuraxis: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs).^{13,14,30,89,128} iGluRs are ligand-gated ion channels that mediate fast synaptic transmission, whereas mGluRs are GPCRs that modulate neuronal excitability through activation of secondary signal transduction pathways. In the periphery, glutamate is released from primary afferents as well as non-neuronal cells in response to tissue damage.^{43,74,123} Upon binding to receptors expressed on peripheral nerve terminals, glutamate contributes to the initiation of nociception as well as sensory neuron sensitization.^{7,14,84} Glutamate also transmits and modulates nociceptive inputs within the SCDH. Specifically, once released from primary afferent presynaptic terminals, glutamate conveys nociceptive signals by binding postsynaptic iGluRs and mGluRs and also regulates subsequent neurotransmitter release by activating presynaptic mGlu autoreceptors.^{14,30,93} Following tissue damage, enhanced activity of iGluRs and mGluRs strengthens excitatory synapses and triggers activity-dependent central sensitization.^{29,93} In supraspinal brain regions such as the thalamus, amygdala, anterior cingulate cortex, and prefrontal cortex, glutamate signaling relays and modifies the somatosensory, emotional, and cognitive aspects of pain.^{14,89} Aberrant glutamate receptor expression and signaling in these brain regions has been implicated in the development and maintenance of chronic pain.^{14,70,117}

Pharmacological inhibition of iGluRs as a treatment for chronic pain has been explored in both preclinical and clinical studies. For instance, antagonists for the *N*-methyl-D-aspartate (NMDA) iGluR subtype were developed with the goal of blocking peripheral and/or central sensitization. Preclinical studies in rodents and horses demonstrated that NMDA antagonists

such as ketamine reduced pain-like behavior, suggesting that they would be viable clinical tools.^{70,117} However, psychotropic properties and dose-limiting negative side effects on learning and memory in a subset set of clinical trials discounted the widespread applicability of targeting NMDA receptors, and more broadly iGluRs, for chronic pain relief.^{32,70} Thus, mGluRs have emerged as favorable alternatives for modulating glutamate signaling in chronic pain states.

1.6.1 Group II mGluRs suppress peripheral sensitization

The mGluRs are a family of seven transmembrane domain GPCRs distinguished by an N-terminus extracellular Venus-flytrap (VFT) domain and extracellular cysteine rich domains (CRDs). The VFT domain houses the binding site for glutamate and other orthosteric ligands, whereas CRDs transmit activation signals to docked G protein complexes.¹⁵⁹ The mGluR family has been subdivided into 3 groups based upon similarities in sequence, pharmacology, and intracellular signaling.³⁶ Group I mGluRs (mGluR1 & 5) are G_q -coupled and lead to phospholipase C activation, in turn increasing neuronal excitability. In contrast, group II (mGluR2 & 3) and group III (mGluR 4,6,7, & 8) mGluRs are both G_i -coupled and suppress neuronal excitability by way of inhibiting adenylyl cyclase, as well as voltage-gated calcium channels, and activating potassium channels.³⁶

A variety of studies have evaluated group I, II, and III mGluRs in rodent models of inflammatory and neuropathic pain. Cumulative evidence suggests that pharmacological inhibition and activation of group I and group II/III mGluRs, respectively, reduces pain-like behavior.^{29,89,115} Though group III mGluRs are now gaining more interest, the group II receptors have been more extensively studied as putative analgesic targets. To elaborate, systemic pharmacological activation of group II mGluRs has been shown to decrease pain-like behavior in rodent models of both inflammatory and neuropathic pain.^{31,79,124,137,139} Interestingly, in uninjured rodents, group II agonists do not alter pain behavior,^{47,79,167} indicating that the

analgesic effect is specific to the sensitized state. While most group II ligands are not selective for mGluR2 versus mGluR3, genetic approaches have been used to distinguish whether activity of mGluR2, mGluR3, or both receptors underlies the analgesic properties of group II agonists. Importantly, analgesic efficacy of group II agonists persists in mGluR3^{-/-}, but not mGluR2^{-/-} mice, suggesting a greater role for mGluR2 than mGluR3 in pain regulation.¹⁶⁸

Following the observation that systemic mGluR2/3 activation reduces rodent pain-like behavior, the site of mGluR2/3 activation along the pain neuraxis required to elicit analgesia became an intriguing question. A variety of findings from our lab and others suggest that mGluR2/3 are well-positioned to depress pain transmission within sensory neurons. Foremost, pharmacological studies targeting sensory neuron mGluR2/3 via intraplantar hindpaw injections demonstrate that peripheral mGluR2/3 activation attenuates rodent nocifensive behavior.^{3,24,167} Conversely, peripheral mGluR2/3 inhibition prolongs nocifensive behavior in rodent hindpaw inflammatory pain models, suggesting that endogenous activation of group II mGluRs is analgesic.^{24,167} Further, anatomical studies confirm expression of mGluR2/3 in rodent sensory neuron peripheral terminals, as well as DRG cell bodies and central projections.^{21-23,40} Lastly, *in vitro* studies of cultured rodent sensory neurons and skin-nerve preparations demonstrate functional expression mGluR2/3 in sensory neurons, as mGluR2/3 activation blocks sensory neuron hyperexcitability.^{23,40,165,166} Our recent studies in human sensory neurons confirm mGluR2/3 anatomical and functional expression across species.⁴⁰ These findings are particularly exciting as mGluR2/3 now represent putative peripheral analgesic targets that could bypass centrally mediated side effects observed with existing analgesics. However, to remain viable analgesic targets, the molecular mechanisms underlying mGluR2/3-induced suppression of sensory neuron hyperexcitability require further investigation.

There are a number of mechanisms by which mGluR2/3 activation within sensory neurons can reduce nociception. Rodent studies demonstrate that on sensory neuron central

terminals within the SC, mGluR2/3 activation negatively regulates neurotransmitter release onto secondary dorsal horn neurons.⁵⁹ Further, on sensory neuron peripheral terminals innervating the skin, mGluR2/3 activation suppresses TRPV1 sensitization elicited by inflammatory mediators such as PGE₂ by inhibiting adenylyl cyclase and the cAMP/PKA pathway (Fig. 2).^{23,165–167} Whether the same mechanisms of peripheral analgesia are conserved in human sensory neurons is investigated in Chapter 3.

1.7 Preclinical approaches to modeling and measuring pain and nociception:

Translational opportunities

Much of our present understanding of nociception as well as physiological and pathological pain can be attributed to the use of preclinical animal models. Clinical studies are frequently complicated by human genetic diversity, differences in chronic pain etiologies, and lack of control over the plethora of environmental conditions that can influence pain perception and pharmacological outcomes such as diet and lifestyle.⁶⁸ In many ways, rodent models minimize these complications.

We now have great command over rodent genetics whereby inbred strains allow for assessment of nociception and pain manipulations in genetically identical test subjects and molecular tools allow us to genetically modify mice with relative ease. For instance, the Cre-LoxP recombination system permits cell-type specific deletion of a gene of interest and analysis of the consequences of such deletions.¹¹⁸ We have previously used this system to eliminate expression of Ret, the GDNF family of ligands receptor, exclusively from nociceptors and demonstrated that Ret is required for the survival and normal function of nonpeptidergic nociceptors.⁶² Besides nociceptor physiology, genetic recombination systems have allowed us to probe within the spinal and central circuits that underlie pain processing with neuron subtype specificity that was unfathomable only three decades ago.¹⁴⁹

1.7.1 Rodent pain models and traditional readouts of pain-like behavior

In addition to genetic control, rodent injury models allow us to investigate injuries with standardized onset, etiology, and location in a controlled environment. Common rodent acute and persistent pain models, including those utilized within the present manuscript, often involve injury to the hindpaw or the nerves innervating the hindpaw. For instance, proinflammatory agents such as formalin or Complete Freund's Adjuvant are injected subdermally into the plantar surface of the hindpaw to model acute and persistent inflammation, respectively.¹³⁰ Nerve injury models including the spared nerve injury model involve either ligation or compression of the nerve or nerve roots comprising the sciatic nerve.^{42,46} Although these rodent pain models represent convenient, controlled manipulations for researchers to investigate putative pharmacological targets for pain relief, their relevance to human chronic pain conditions has been questioned.^{33,101,112} The incorporation of diabetic- and chemotherapy-induced neuropathy as well as metastatic bone cancer pain models represents one attempt to increase the clinical applicability of preclinical pain research^{33,127}; however these models do not fully recapitulate human disease and have not replaced traditional inflammation and nerve injury models.

The clinical relevance of traditional pain behavioral measures has also received great scrutiny. Preclinical pain researchers have historically relied on measures of nociceptive withdrawal reflexes: a mechanical, heat, or cold stimulus is applied to the plantar surface of the hindpaw and the withdrawal response threshold and/or latency is reported. Injury-induced hypersensitivity is indicated by reduced response thresholds/latencies. In some injury models such as the formalin test, spontaneous nocifensive behaviors including licking and lifting of the hindpaw can be quantified. Withdrawal reflex and spontaneous nocifensive behavioral endpoints encompass the somatosensory component of pain. However, the human pain experience is a complex sensory, cognitive, and emotional experience, and the primary

outcome in clinical trials is often improved health-related quality of life measures rather than improved pain ratings.^{122,145} This mismatch between rodent and human pain measures is proposed to contribute to the poor translational record of putative analgesics identified in rodent models.^{6,33,34,112,113,131,134,148} Thus, the development of voluntary and quality of life behavioral measures in rodents that reflect the complexities of human pain could improve the translation of basic pain research.

1.7.2 In vitro approaches towards understanding nociception

In vitro rodent models have provided an excellent foundation in our understanding of the molecular mechanisms that mediate nociception and peripheral sensitization. For example, cultured primary sensory neurons and semi-intact nerve preparations can be used to identify neuronal subpopulations that convey distinct somatosensory modalities.^{5,65,153,154} These insights are certainly informative, yet despite the evolutionary significance of pain, rodents are not small, fuzzy people. Rather, rodents and humans undoubtedly possess distinct pain-mediating molecular palettes. The voltage-gated sodium channel Na_v1.7 represents one notable example of species differences in nociceptor physiology. Humans lacking the voltage-gated sodium channel Na_v1.7 are unable to detect any form of pain, whereas only some pain phenotypes are lost in nociceptor-specific Na_v1.7-conditional knock out mice.^{7,37,111,119} Therefore, verifying that molecular targets/mechanisms identified in rodents are conserved in human tissue prior to conducting clinical studies could immensely improve the translational record of preclinical analgesics. Limited access to human tissue has unfortunately obstructed these investigations. To address this limitation, we and others have sought access to human sensory neurons.^{39,51,173} In collaboration with Mid-America Transplant, we recently established a protocol to surgically extract human DRG from deceased organ donors and culture sensory neurons from these

donors.¹⁵⁵ Thus, we now have the exciting and unique opportunity to study human nociceptor anatomy and physiology.

1.8 Approaches for bridging the translation gap between rodent and human pain research

In summary, traditional rodent pain models and behavioral measures provide an important foundational understanding of mechanisms of nociception and pain manipulations. However, to improve the translational record of preclinical analgesics and tackle the public health issue of treating chronic pain, it is critical that we bridge the translational gap between rodent and human pain research. The present manuscript describes three studies in which I aim to increase the translatability of basic pain research. First, I investigate a variety of voluntary behaviors as read outs of persistent pain in mice (Chapter 2). Second, I utilize human and mouse sensory neurons to determine whether the mechanism of mGluR2/3-induced suppression of sensory neuron sensitization is conserved across species (Chapter 3). Finally, I evaluate whether voluntary exercise in rodents can be used as a model to elucidate the mechanisms that underlie the clinical observation that exercise is an effective nonpharmacological intervention for pain relief (Chapter 4).

1.9 References

1. Abaira VE, Ginty DD: The sensory neurons of touch. *Neuron* 79:618–39, 2013.
2. Almeida TF, Roizenblatt S, Tufik S: Afferent pain pathways: a neuroanatomical review. *Brain Res* 1000:40–56, 2004.
3. Asseri KA, Puil E, Schwarz SKW, MacLeod BA: Group II metabotropic glutamate receptor antagonism prevents the antiallodynic effects of R-isovaline. *Neuroscience* 293:151–6, 2015.
4. Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ, Patapoutian A: Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* 41:849–57, 2004.
5. Barabas ME, Stucky CL: TRPV1, but not TRPA1, in primary sensory neurons contributes to cutaneous incision-mediated hypersensitivity. *Mol Pain* 9:9, 2013.
6. Barrot M: Tests and models of nociception and pain in rodents. *Neuroscience* 211:39–50, 2012.
7. Basbaum AI, Bautista DM, Scherrer G, Julius D: Cellular and molecular mechanisms of pain. *Cell* 139:267–84, 2009.
8. Basso L, Altier C: Transient Receptor Potential Channels in neuropathic pain. *Curr Opin Pharmacol* 32:9–15, 2017.
9. Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, Julius D: TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents. *Cell* 124:1269–82, 2006.
10. Bautista DM, Lumpkin EA, Bautista DM, Lumpkin EA: Probing mammalian touch transduction. *J Gen Physiol* 138:291–301, 2011.
11. Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt S-E, Julius D: The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* 448:204–8, 2007.
12. Bhawe G, Zhu W, Wang H, Brasier D., Oxford GS, Gereau RW: cAMP-Dependent Protein Kinase Regulates Desensitization of the Capsaicin Receptor (VR1) by Direct Phosphorylation. *Neuron* 35:721–31, 2002.
13. De Blasi A, Conn PJ, Pin J-P, Nicoletti F: Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol Sci* 22:114–20, 2001.
14. Bleakman D, Alt A, Nisenbaum ES: Glutamate receptors and pain. *Semin Cell Dev Biol* 17:592–604, 2006.
15. Blyth FM: Chronic pain - Is it a public health problem? *Pain* 137:465–6, 2008.
16. Bourne S, Machado AG, Nagel SJ: Basic anatomy and physiology of pain pathways. *Neurosurg Clin N Am* 25:629–38, 2014.
17. Brandt K: Non-surgical treatment of osteoarthritis: a half century of “advances.” *Ann*

- Rheum Dis 63:117–23, 2004.
18. Braz JM, Nassar MA, Wood JN, Basbaum AI: Parallel “pain” pathways arise from subpopulations of primary afferent nociceptor. *Neuron* 47:787–93, 2005.
 19. Brown DC: Resiniferatoxin: The evolution of the “molecular scalpel” for chronic pain relief. *Pharmaceuticals* 9:1–11, 2016.
 20. Campbell JN, Raja SN, Meyer R a, Mackinnon SE: Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain* 32:89–94, 1988.
 21. Carlton S, Hargett G: Colocalization of Metabotropic Glutamate Receptors in Rat Dorsal Root Ganglion Cells. *J Comp Neurol* 501:780–9, 2007.
 22. Carlton S, Hargett G, Coggeshall R: Localization of metabotropic glutamate receptors 2/3 on primary afferent axons in the rat. *Neuroscience* 105:957–69, 2001.
 23. Carlton SM, Du J, Zhou S: Group II metabotropic glutamate receptor activation on peripheral nociceptors modulates TRPV1 function. *Brain Res* 1248:86–95, 2009.
 24. Carlton SM, Zhou S, Govea R, Du J: Group II/III Metabotropic Glutamate Receptors Exert Endogenous Activity-Dependent Modulation of TRPV1 Receptors on Peripheral Nociceptors. *J Neurosci* 31:12727–37, 2011.
 25. Caterina MJ, Leffler A, Malmberg AB, Martin W, Trafton J, Petersen-Zeit K, Koltzenburg M, Basbaum AI, Julius D: Impaired Nociception and Pain Sensation in Mice Lacking the Capsaicin Receptor. *Science* (80-) 288:306–13, 2000.
 26. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D: The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816–24, 1997.
 27. Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ: Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc Natl Acad Sci U S A* 106:9075–80, 2009.
 28. Cheng J-K, Ji R-R: Intracellular signaling in primary sensory neurons and persistent pain. *Neurochem Res* 33:1970–8, 2008.
 29. Chiechio S: Modulation of Chronic Pain by Metabotropic Glutamate Receptors. *Adv Pharmacol* 75:63–89, 2016.
 30. Chiechio S, Copani A, Zammataro M, Battaglia G, Gereau RW, Nicoletti F: Transcriptional regulation of type-2 metabotropic glutamate receptors: an epigenetic path to novel treatments for chronic pain. *Trends Pharmacol Sci* 31:153–60, 2010.
 31. Chiechio S, Zammataro M, Morales ME, Busceti CL, Drago F, Gereau RW, Copani A, Nicoletti F: Epigenetic Modulation of mGlu2 Receptors by Histone Deacetylase Inhibitors in the Treatment of Inflammatory Pain. *Mol Pharmacol* 75:1014–20, 2009.
 32. Chizh BA: Low dose ketamine: a therapeutic and research tool to explore N-methyl-D-aspartate (NMDA) receptor-mediated plasticity in pain pathways. *J Psychopharmacol* 21:259–71, 2007.

33. Clark JD: Preclinical Pain Research: Can We Do Better? *Anesthesiology* 125:846–9, 2016.
34. Cobos EJ, Portillo-Salido E: “Bedside-to-Bench” Behavioral Outcomes in Animal Models of Pain: Beyond the Evaluation of Reflexes. *Curr Neuropharmacol* 11:560–91, 2013.
35. Colburn RW, Lubin M Lou, Stone DJ, Wang Y, Lawrence D, D’Andrea MR, Brandt MR, Liu Y, Flores CM, Qin N: Attenuated cold sensitivity in TRPM8 null mice. *Neuron* 54:379–86, 2007.
36. Conn PJ, Pin JP: Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol* 37:205–37, 1997.
37. Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG: An SCN9A channelopathy causes congenital inability to experience pain. *Nature* 444:894–8, 2006.
38. Crawford C, Buckenmaier C, Schoomaker E, Delgado R, York A: Active, Self-Care Complementary and Integrative Medicine Therapies for the Management of Chronic Pain Symptoms: A Rapid Evidence Assessment of the Literature. *J Altern Complement Med* 20:A137–8, 2014.
39. Davidson S, Copits BA, Zhang J, Page G, Ghetti A, Gereau RW: Human sensory neurons: Membrane properties and sensitization by inflammatory mediators. *Pain* 155:1861–70, 2014.
40. Davidson S, Golden JP, Copits BA, Ray PR, Vogt SK, Price TJ, Schmidt RE, Ghetti A, Gereau RW: Group 2 mGluRs suppress hyperexcitability in mouse and human nociceptors. *Pain* 157:2081–8, 2016.
41. Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA: Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 405:183–7, 2000.
42. Decosterd I, Woolf CJ: Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87:149–58, 2000.
43. deGroot J, Zhou S, Carlton SM: Peripheral glutamate release in the hindpaw following low and high intensity sciatic stimulation. *Neuroreport* 11:497–502, 2000.
44. Devor M: Ectopic discharge in AB afferents as a source of neuropathic pain. *Exp Brain Res* 196:115–28, 2009.
45. Dhaka A, Murray AN, Mathur J, Earley TJ, Petrus MJ, Patapoutian A: TRPM8 is required for cold sensation in mice. *Neuron* 54:371–8, 2007.
46. Dowdall T, Robinson I, Meert TF: Comparison of five different rat models of peripheral nerve injury. *Pharmacol Biochem Behav* 80:93–108, 2005.
47. Du J, Zhou S, Carlton SM: Group II metabotropic glutamate receptor activation attenuates peripheral sensitization in inflammatory states. *Neuroscience* 154:754–66,

2008.

48. Dubin AE, Patapoutian A: Nociceptors: The sensors of the pain pathway. *J Clin Invest* 120:3760–72, 2010.
49. Dysvik E, Lindstrøm TC, Eikeland OJ, Natvig GK: Health-related quality of life and pain beliefs among people suffering from chronic pain. *Pain Manag Nurs* 5:66–74, 2004.
50. Everill B, Kocsis JD, Stemkowski PL, Smith PA: Reduction in Potassium Currents in Identified Cutaneous Afferent Dorsal Root Ganglion Neurons After Axotomy Reduction in Potassium Currents in Identified Cutaneous Afferent Dorsal Root Ganglion Neurons After Axotomy. *J Neurophysiol* 82:700–8, 1999.
51. Facer P, Casula MA, Smith GD, Benham CD, Chessell IP, Bountra C, Sinisi M, Birch R, Anand P: Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4 and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy. *BMC Neurol* 7:11, 2007.
52. Feldman JJ, Bowman EN, Phillips BB, Weinlein JC: Tibial Stress Fractures in Athletes Tibia Stress fracture Athlete Intramedullary fixation Tension band plate. *Orthop Clin NA* 47:733–41, 2016.
53. Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL: Sex, Gender, and Pain: A Review of Recent Clinical and Experimental Findings. *J Pain* 10:447–85, 2009.
54. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, Gilron I, Haanpaa M, Hansson P, Jensen TS, Kamerman PR, Lund K, Moore A, Raja SN, Rice ASC, Rowbotham M, Sena E, Siddall P, Smith BH, Wallace M: Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. *Lancet Neurol* 14:162–73, 2015.
55. Finnerup NB, Sindrup SH, Jensen TS: The evidence for pharmacological treatment of neuropathic pain. *Pain* 150:573–81, 2010.
56. Gavva NR, Treanor JJS, Garami A, Fang L, Surapaneni S, Akrami A, Alvarez F, Bak A, Darling M, Gore A, Jang GR, Kesslak JP, Ni L, Norman MH, Palluconi G, Rose MJ, Salfi M, Tan E, Romanovsky AA, Banfield C, Davar G: Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* 136:202–10, 2008.
57. Geffeney SL, Goodman MB: How We Feel: Ion Channel Partnerships that Detect Mechanical Inputs and Give Rise to Touch and Pain Perception. *Neuron*. page 609–192012.
58. Geneen L, Smith B, Clarke C, Martin D, Colvin LA, Moore RA: Physical activity and exercise for chronic pain in adults: an overview of Cochrane reviews. *Cochrane Database Syst Rev* :11279, 2014.
59. Gerber G, Zhong J, Youn D, Randic M: Group II and group III metabotropic glutamate receptor agonists depress synaptic transmission in the rat spinal cord dorsal horn. *Neuroscience* 100:393–406, 2000.
60. Gereau RW, Sluka KA, Maixner W, Savage SR, Price TJ, Murinson BB, Sullivan MD,

- Fillingim RB: A pain research agenda for the 21st century. *J Pain* 15:1203–14, 2014.
61. Goldberg DS, Mcgee SJ: Pain as a global public health priority. *BMC Public Health* 11:1–5, 2011.
 62. Golden JP, Hoshi M, Nassar MA, Enomoto H, Wood JN, Milbrandt J, Gereau RW, Johnson EM, Jain S: RET signaling is required for survival and normal function of nonpeptidergic nociceptors. *J Neurosci* 30:3983–94, 2010.
 63. Gracely RH, Lynch SA, Bennett GJ: Painful neuropathy: altered central processing maintained dynamically by peripheral input. *Pain* 51:175–94, 1992.
 64. Gureje O: Comorbidity of pain and anxiety disorders. *Curr Psychiatry Rep* 10:318–22, 2008.
 65. Hachisuka J, Baumbauer KM, Omori Y, Snyder LM, Koerber HR, Ross SE: Semi-intact ex vivo approach to investigate spinal somatosensory circuits. *Elife* 5:1–19, 2016.
 66. Hagains CE, Trevino LA, He J, Liu H, Peng YB: Contributions of dorsal root reflex and axonal reflex to formalin-induced inflammation. *Brain Res* 1359:90–7, 2010.
 67. He W, Goodkind D, Kowal P: An Aging World : 2015 International Population Reports. 2016.
 68. von Hehn CA, Baron R, Woolf CJ: Review Deconstructing the Neuropathic Pain Phenotype to Reveal Neural Mechanisms. *Neuron* 73:638–52, 2012.
 69. Heiss J, Iadarola M, Cantor F, Oughourli A, Smith R, Mannes A: A Phase I study of the intrathecal administration of resiniferatoxin for treating severe refractory pain associated with advanced cancer. *J Pain* 15:S67, 2014.
 70. Hewitt D: The Use of NMDA-Receptor Antagonists in the Treatment of Chronic Pain. *Clin. J. Pain.* page S73–92000.
 71. Hong S, Wiley JW: Early painful diabetic neuropathy is associated with differential changes in the expression and function of vanilloid receptor 1. *J Biol Chem* 280:618–27, 2005.
 72. Hu H-J, Bhawe G, Gereau RW: Prostaglandin and protein kinase A-dependent modulation of vanilloid receptor function by metabotropic glutamate receptor 5: potential mechanism for thermal hyperalgesia. *J Neurosci* 22:7444–52, 2002.
 73. Hu J, Milenkovic N, Lewin GR: The high threshold mechanotransducer: A status report. *Pain* 120:3–7, 2006.
 74. Inquimbert P, Bartles K, Olusegun BB, Barret LB, Tegeder I, Scholz J: Peripheral nerve injury produces a sustained shift in the balance between glutamate release and uptake in the dorsal horn of the spinal cord. *Pain* 153:2422–31, 2012.
 75. Institute of Medicine: Relieving Pain in America : A Blueprint for Transforming Prevention, Care, Education, and Research. 2011.
 76. Jensen M, Chodroff M, Dworkin R: The impact of neuropathic pain on health-related quality of life: review and implications. *Neurology* 68:1178–82, 2007.

77. Ji R-R, Samad TA, Jin S-X, Schmoll R, Woolf CJ: p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 36:57–68, 2002.
78. Jin X, Morsy N, Winston J, Pasricha PJ, Garrett K, Akbarali HI: Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase. *Am J Physiol Cell Physiol* 287:C558–63, 2004.
79. Jones CK, Eberle EL, Peters SC, Monn J a, Shannon HE: Analgesic effects of the selective group II (mGlu2/3) metabotropic glutamate receptor agonists LY379268 and LY389795 in persistent and inflammatory pain models after acute and repeated dosing. *Neuropharmacology* 49:206–18, 2005.
80. Julius D: TRP channels and pain. *Annu Rev Cell Dev Biol* 29:355–84, 2013.
81. Kanai Y, Nakazato E, Fujiuchi A, Hara T, Imai A: Involvement of an increased spinal TRPV1 sensitization through its up-regulation in mechanical allodynia of CCI rats. *Neuropharmacology* 49:977–84, 2005.
82. Kanouse AB, Compton P: The Epidemic of Prescription Opioid Abuse, the Subsequent Rising Prevalence of Heroin Use, and the Federal Response. *J Pain Palliat Care Pharmacother* 29:102–14, 2015.
83. Karashima Y, Talavera K, Everaerts W, Janssens A, Kwan KY, Vennekens R, Nilius B, Voets T: TRPA1 acts as a cold sensor in vitro and in vivo. *Proc Natl Acad Sci* 106:1273–8, 2009.
84. Karim F, Bhawe G, Gereau RW: Metabotropic glutamate receptors on peripheral sensory neuron terminals as targets for the development of novel analgesics. *Mol Psychiatry* 6:615–7, 2001.
85. Kim SE, Coste B, Chadha A, Cook B, Patapoutian A: The role of Drosophila Piezo in mechanical nociception. *Nature* 483:209–12, 2012.
86. Kissin I: The development of new analgesics over the past 50 years: A lack of real breakthrough drugs. *Anesth Analg* 110:780–9, 2010.
87. Knowlton WM, Bifolck-Fisher A, Bautista DM, McKemy DD: TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics in vivo. *Pain* 150:340–50, 2010.
88. Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A, Noguchi K: Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with A δ /C-fibers and colocalization with Trk receptors. *J Comp Neurol* 493:596–606, 2005.
89. Kolber BJ: mGluRs Head to Toe in Pain. *Prog Mol Biol Transl Sci* 131:281–324, 2015.
90. Von Korff MR: Long-term use of opioids for complex chronic pain. *Best Pract Res Clin Rheumatol* 27:663–72, 2013.
91. Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ, Corey DP: TRPA1 Contributes to Cold, Mechanical, and Chemical Nociception but Is Not Essential for Hair-Cell Transduction. *Neuron* 50:277–89, 2006.
92. Kwan KY, Corey DP: Burning Cold: Involvement of TRPA1 in Noxious Cold Sensation. *J*

- Gen Physiol 133:251–6, 2009.
93. Latremoliere A, Woolf CJ: Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity. *J Pain* 10:895–926, 2009.
 94. Leach RE, Purnell M, Saito A: Peroneal nerve entrapment in runners. *Am J Sports Med* 17:287–91, 1988.
 95. Lee JY, Shin TJ, Choi JM, Seo KS, Kim HJ, Yoon TG, Lee YS, Han H, Chung HJ, Oh Y, Jung SJ, Shin KJ: Antinociceptive curcuminoid, KMS4034, effects on inflammatory and neuropathic pain likely via modulating TRPV1 in mice. *Br J Anaesth* 111:667–72, 2013.
 96. Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M: Tactile allodynia in the absence of C-fiber activation: Altered firing properties of DRG neurons following spinal nerve injury. *Pain* 85:503–21, 2000.
 97. Loeser JD, Treede RD: The Kyoto protocol of IASP Basic Pain Terminology. *Pain* 137:473–7, 2008.
 98. Lopshire JC, Nicol GD: The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies. *J Neurosci* 18:6081–92, 1998.
 99. Louis SM, Jamieson A, Russel W, Dockray GJ: The role of substance p and calcitonin gene-related peptide in neurogenic plasma extravasation and vasodilatation in the rat. *Neuroscience* 32:581–6, 1989.
 100. Mannes A, Hughes M, Quezada Z, Berger A, Fojo T, Smith R, Butman J, Lonser R, Iadarola M: Resiniferatoxin, a potent TRPV1 agonist: Intrathecal administration to treat severe pain associated with advanced cancer - Case report. *J Pain* 11:S43, 2010.
 101. Mao J: Current challenges in translational pain research. *Trends Pharmacol Sci* 33:568–73, 2012.
 102. Matcuk GR, Mahanty SR, Skalski MR, Patel DB, White EA, Gottsegen CJ: Stress fractures : pathophysiology, clinical presentation, imaging features, and treatment options. *Emerg Radiol* :365–75, 2016.
 103. McCoy ES, Taylor-Blake B, Street SE, Pribisko AL, Zheng J, Zylka MJ: Peptidergic CGRP Primary Sensory Neurons Encode Heat and Itch and Tonically Suppress Sensitivity to Cold. *Neuron* 78:138–51, 2013.
 104. Mckemy DD: How cold is it? TRPM8 and TRPA1 in the molecular logic of cold sensation. *Mol Pain* 1:16, 2005.
 105. McKemy DD, Neuhausser WM, Julius D: Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* 416:52–8, 2002.
 106. Merighi A: Capsaicin, Nociception and Pain. *Molecules* 21:797, 2016.
 107. Meves H: The action of prostaglandins on ion channels. *Curr Neuropharmacol* 4:41–57, 2006.
 108. Michaelis M, Häbler HJ, Jäenig W, Habler H-J, Janig W: Silent afferents: A separate

- class of primary afferents? *Clin Exp Pharmacol Physiol* 23:99–105, 1996.
109. Mickle AD, Shepherd AJ, Mohapatra DP: Sensory TRP channels: the key transducers of nociception and pain. *Prog Mol Biol Transl Sci* 131:73–118, 2015.
 110. Mifflin KA, Kerr BJ: The transition from acute to chronic pain: Understanding how different biological systems interact. *Can J Anesth* 61:112–22, 2014.
 111. Minett MS, Falk S, Santana-Varela S, Bogdanov YD, Nassar MA, Heegaard AM, Wood JN: Pain without nociceptors? Nav1.7-independent pain mechanisms. *Cell Rep* 6:301–12, 2014.
 112. Mogil JS: Animal models of pain: progress and challenges. *Nat Rev Neurosci* 10:283–94, 2009.
 113. Mogil JS, Cragger SE: What should we be measuring in behavioral studies of chronic pain in animals? *Pain* 112:12–5, 2004.
 114. Mohapatra DP, Nau C: Desensitization of Capsaicin-activated Currents in the Vanilloid Receptor TRPV1 Is Decreased by the Cyclic AMP-dependent Protein Kinase Pathway. *J Biol Chem* 278:50080–90, 2003.
 115. Montana MC, Gereau RW: Metabotropic glutamate receptors as targets for analgesia: antagonism, activation, and allosteric modulation. *Curr Pharm Biotechnol* 12:1681–8, 2011.
 116. Moore A, Derry S, Eccleston C, Kalso E: Expect analgesic failure; pursue analgesic success. *Br Med J* 346:f2690, 2013.
 117. Muir WW: NMDA Receptor Antagonists and Pain: Ketamine. *Vet Clin North Am - Equine Pract* 26:565–78, 2010.
 118. Nagy A: Cre recombinase: The universal reagent for genome tailoring. *Genesis* 26:99–109, 2000.
 119. Nassar MA, Levato A, Stirling LC, Wood JN: Neuropathic pain develops normally in mice lacking both Na(v)1.7 and Na(v)1.8. *Mol Pain* 1:24, 2005.
 120. Nicholson B, Verma S: Comorbidities in chronic neuropathic pain. *Pain Med* 5:S9–27, 2004.
 121. Nilius B, Szallasi A: Transient receptor potential channels as drug targets: from the science of basic research to the art of medicine. *PharmacolRev* 66:676–814, 2014.
 122. O'Connor AB: Neuropathic Pain: Quality-of-Life Impact, Costs, and Cost Effectiveness of Therapy. *Pharmacoeconomics* 27:95–112, 2009.
 123. Omote K, Kawamata T, Kawamata M, Namiki A: Formalin-induced release of excitatory amino acids in the skin of the rat hindpaw. *Brain Res* 787:161–4, 1998.
 124. Osikowicz M, Mika J, Makuch W, Przewlocka B: Glutamate receptor ligands attenuate allodynia and hyperalgesia and potentiate morphine effects in a mouse model of neuropathic pain. *Pain* 139:117–26, 2008.

125. Patel K V, Guralnik JM, Dansie EJ, Turk DC: Prevalence and impact of pain among older adults in the United States : Findings from the 2011 National Health and Aging Trends Study. *Pain* 154:2649–57, 2013.
126. Peirs C, Seal RP: Neural circuits for pain: Recent advances and current views. *Science* (80-) 35:578–84, 2016.
127. Percie Du Sert N, Rice ASC: Improving the translation of analgesic drugs to the clinic: Animal models of neuropathic pain. *Br J Pharmacol* 171:2951–63, 2014.
128. Pin J-P, Duvoisin R: The metabotropic glutamate receptors: Structure and functions. *Neuropharmacology* 34:1–26, 1995.
129. Rashid MH, Inoue M, Kondo S, Kawashima T, Bakoshi S, Ueda H: Novel expression of vanilloid receptor 1 on capsaicin-insensitive fibers accounts for the analgesic effect of capsaicin cream in neuropathic pain. *J Pharmacol Exp Ther* 304:940–8, 2003.
130. Ren K, Dubner R: Inflammatory Models of Pain and Hyperalgesia. *ILAR J* 40:111–8, 1999.
131. Rice ASC, Cimino-Brown D, Eisenach JC, Kontinen VK, Lacroix-Fralish ML, Machin I, Mogil JS, Stoehr T: Comment on: Animal models and the prediction of efficacy in clinical trials of analgesic drugs: A critical appraisal and a call for uniform reporting standards. *Pain* 139:243–7, 2009.
132. Ringkamp M, Raja SN, Campbell JN, Meyer RA: Peripheral Mechanisms of Cutaneous Nociception. In: McMahon S, Koltzenburg M, Tracey I, Turk DC, editors. *Wall Melzack's Textb Pain* 6th ed. page 1–30 2013.
133. Sabatowski R, Schäfer D, Kasper SM, Brunsch H, Radbruch L: Pain treatment: a historical overview. *Curr Pharm Des* 10:701–16, 2004.
134. Sapunar D, Puljak L: What can rats tell us about neuropathic pain? Critical evaluation of behavioral tests used in rodent pain models. *Period Biol* 111:155–60, 2009.
135. Schaible H-G: Peripheral and Central Mechanisms of Pain Generation. *Analgesia* 177:3–28, 2006.
136. Schouenborg J, Kalliomäki J: Functional organization of the nociceptive withdrawal reflexes - I. Activation of hindlimb muscles in the rat. *Exp Brain Res* 83:67–78, 1990.
137. Sharpe EF, Kingston AE, Lodge D, Monn JA, Headley PM: Systemic pre-treatment with a group II mGlu agonist, LY379268, reduces hyperalgesia in vivo. *Br J Pharmacol* 135:1255–62, 2002.
138. Sherrington CS: Qualitative difference of spinal reflex corresponding with qualitative difference of cutaneous stimulus. *J Physiol* 30:39–46, 1903.
139. Simmons RMA, Webster AA, Kalra AB, Iyengar S: Group II mGluR receptor agonists are effective in persistent and neuropathic pain models in rats. *Pharmacol Biochem Behav* 73:419–27, 2002.
140. Smith-Edwards KM, Deberry JJ, Saloman JL, Davis BM, Jeffery Woodbury C: Profound alteration in cutaneous primary afferent activity produced by inflammatory mediators. *Elife*

5:1–18, 2016.

141. Southall MD, Vasko MR: Prostaglandin Receptor Subtypes, EP3C and EP4, Mediate the Prostaglandin E2-induced cAMP Production and Sensitization of Sensory Neurons. *J Biol Chem* 276:16083–91, 2001.
142. St E, Lewin GR: Nociceptors: a phylogenetic view. *J Comp Physiol* 195:1089–106, 2009.
143. Stein C: Opioids, sensory systems and chronic pain. *Eur J Pharmacol* 716:179–87, 2013.
144. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A: ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 112:819–29, 2003.
145. Sullivan MD, Ballantyne JC: Must we reduce pain intensity to treat chronic pain? *Pain* 157:65–9, 2016.
146. Sun Q, Tu H, Xing GG, Han JS, Wan Y: Ectopic discharges from injured nerve fibers are highly correlated with tactile allodynia only in early, but not late, stage in rats with spinal nerve ligation. *Exp Neurol* 191:128–36, 2005.
147. Szallasi A, Sheta M: Targeting TRPV1 for pain relief: limits, losers and laurels. *Expert Opin Investig Drugs* 21:1351–69, 2012.
148. Tappe-Theodor A, Kuner R: Studying ongoing and spontaneous pain in rodents - challenges and opportunities. *Eur J Neurosci* 39:1881–90, 2014.
149. Todd AJ: Identifying functional populations among the interneurons in laminae I-III of the spinal dorsal horn. *Mol Pain* 13:174480691769300, 2017.
150. Touska F, Marsakova L, Teisinger J, Vlachova V: A “cute” desensitization of TRPV1. *Curr Pharm Biotechnol* 12:122–9, 2011.
151. Tsang A, Von Korff M, Lee S, Alonso J, Karam E, Angermeyer MC, Borges GLG, Bromet EJ, de Girolamo G, de Graaf R, Gureje O, Lepine J, Haro JM, Levinson D, Oakley Browne MA, Posada-Villa J, Seedat S, Watanabe M: Common Chronic Pain Conditions in Developed and Developing Countries: Gender and Age Differences and Comorbidity With Depression-Anxiety Disorders. *J Pain* 9:883–91, 2008.
152. Urano H, Ara T, Fujinami Y, Hiraoka BY: Aberrant TRPV1 expression in heat hyperalgesia associated with trigeminal neuropathic pain. *Int J Med Sci* 9:690–7, 2012.
153. Valtcheva M V, Davidson S, Zhao C, Leitges M, Gereau RW: Protein kinase C δ mediates histamine-evoked itch and responses in pruriceptors. *Mol Pain* 11:1, 2015.
154. Valtcheva M V, Samineni VK, Golden JP, Gereau RW, Davidson S: Enhanced Nonpeptidergic Intraepidermal Fiber Density and an Expanded Subset of Chloroquine-Responsive Trigeminal Neurons in a Mouse Model of Dry Skin Itch. *J Pain* 16:346–56, 2015.
155. Valtcheva MV, Copits BA, Davidson S, Sheahan TD, Pullen MY, McCall JG, Dikranian K, Gereau RW: Surgical extraction of human dorsal root ganglia from organ donors and preparation of primary sensory neuron cultures. *Nat Protoc* 11:, 2016.

156. Wallace LS, Wexler RK, McDouggle L, Miser WF, Haddox JD: Voices that may not otherwise be heard: A qualitative exploration into the perspectives of primary care patients living with chronic pain. *J Pain Res* 7:291–9, 2014.
157. Walsh CM, Bautista DM, Lumpkin EA: Mammalian touch catches up. *Curr Opin Neurobiol* 34:133–9, 2015.
158. Whiteside GT, Kennedy J: Consideration of Pharmacokinetic Pharmacodynamic Relationships in the Discovery of New Pain Drugs. Kruger L, Light A, editors. *Transl. Pain Res. From Mouse to Man*. 2010.
159. Willard SS, Koochekpour S: Glutamate, glutamate receptors, and downstream signaling pathways. *Int J Biol Sci* 9:948–59, 2013.
160. Willis WD: Dorsal root potentials and dorsal root reflexes : a double-edged sword. *Exp Brain Res* 124:395–421, 1999.
161. Woolf CJ, Ma Q: Nociceptors — Noxious Stimulus Detectors. *Neuron* 55:353–64, 2007.
162. Wu G, Ringkamp M, Hartke T V, Murinson BB, Campbell JN, Griffin JW, Meyer R a: Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers. *J Neurosci* 21:21, 2001.
163. Xu GY, Huang LY, Zhao ZQ: Activation of silent mechanoreceptive cat C and Adelta sensory neurons and their substance P expression following peripheral inflammation. *J Physiol* 528 Pt 2:339–48, 2000.
164. Xu Z-Z, Kim YH, Bang S, Zhang Y, Berta T, Wang F, Oh SB, Ji R-R: Inhibition of mechanical allodynia in neuropathic pain by TLR5-mediated A-fiber blockade. *Nat Med* 21:1326–31, 2015.
165. Yang D, Gereau RW: Group II metabotropic glutamate receptors inhibit cAMP-dependent protein kinase-mediated enhancement of tetrodotoxin-resistant sodium currents in mouse dorsal root ganglion neurons. *Neurosci Lett* 357:159–62, 2004.
166. Yang D, Gereau RW: Peripheral group II metabotropic glutamate receptors (mGluR2/3) regulate prostaglandin E2-mediated sensitization of capsaicin responses and thermal nociception. *J Neurosci* 22:6388–93, 2002.
167. Yang D, Gereau RW: Peripheral group II metabotropic glutamate receptors mediate endogenous anti-allodynia in inflammation. *Pain* 106:411–7, 2003.
168. Zammataro M, Chiechio S, Montana MC, Traficante A, Copani A, Nicoletti F, Gereau RW: mGlu2 metabotropic glutamate receptors restrain inflammatory pain and mediate the analgesic activity of dual mGlu2/mGlu3 receptor agonists. *Mol Pain* 7:6, 2011.
169. Zappia KJ, O'Hara CL, Moehring F, Kwan KY, Stucky CL: Sensory neuron-specific deletion of TRPA1 results in mechanical cutaneous sensory deficits. *Eneuro* 4:e.0069-16.2017, 2017.
170. Zeidan F, Vago DR: Mindfulness meditation–based pain relief: a mechanistic account. *Ann N Y Acad Sci* 1373:114–27, 2016.
171. Zhang X, Huang J, McNaughton PA: NGF rapidly increases membrane expression of

- TRPV1 heat-gated ion channels. *EMBO J* 24:4211–23, 2005.
172. Zhang X, McNaughton PA: Why pain gets worse: the mechanism of heat hyperalgesia. *J Gen Physiol* 128:491–3, 2006.
 173. Zhang XL, Lee KY, Priest BT, Belfer I, Gold MS: Inflammatory mediator-induced modulation of GABAA currents in human sensory neurons. *Neuroscience* 310:401–9, 2015.
 174. Zylka MJ, Rice FL, Anderson DJ: Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron* 45:17–25, 2005.

Chapter 2

Inflammation and nerve injury minimally affect mouse voluntary behaviors
proposed as indicators of pain

This chapter contains the manuscript:

Sheahan TD, Siuda ER, Bruchas MR, Shepherd AJ, Mohapatra DP, Gereau RW, IV, Golden JP: Inflammation and nerve injury minimally interfere with mouse voluntary behaviors proposed as indicators of pain. *Neurobiology of Pain* 2:1-12, 2017. doi: 10.1016/j.ynpai.2017.09.001

2.1 Abstract

It has been suggested that the lack of rodent behavioral assays that represent the complexities of human pain contributes to the poor translational record of basic pain research findings. Clinically, chronic pain interferes with patient mobility and physical/social activities, and increases anxiety symptoms, in turn negatively impacting quality of life. To determine whether these behaviors are similarly influenced by putative pain manipulations in rodents, we systematically evaluated wheel running, locomotion, gait, social interaction, and anxiety-like behavior in models of inflammation and nerve injury in adult C57BL6/J male mice. We demonstrate that inflammation and nerve injury differentially affect voluntary behaviors while mice are hypersensitive to mechanical stimuli. Bilateral Complete Freund's Adjuvant (CFA)-induced inflammation transiently suppressed wheel running and locomotion and also induced gait deficits. In contrast, spared nerve injury (SNI) altered gait and impaired gross motor coordination. SNI-induced gait changes were not reversed by the analgesic PD123319, an angiotensin II type 2 receptor antagonist, and are therefore likely to be motor-related rather than pain-related. Neither CFA nor SNI significantly altered social interaction or elicited general anxiety-like behavior. Our findings suggest that in contrast to humans, mobility and physical/social activities are minimally altered, if at all, in mice following inflammation or nerve injury.

2.2 Introduction

Chronic pain is an immense clinical and societal burden with largely unsatisfactory pharmacological treatment options.^{22,29,55} Considering the rising opioid epidemic, it is pressing that we develop new, efficacious drug therapies. However, over the past 50 years, the development of novel analgesics has been hindered by the high failure rate of clinical trials.^{32,48} The lack of rodent pain assays that encompass the complexities of human chronic pain is thought to contribute to the poor translational record of preclinical analgesics.^{2,10,13,37,43,45,65,72,85,88} Clinically, chronic pain is characterized by sensory, affective, and emotional changes that negatively impact quality of life.⁴¹ To this end, outcomes of interest in clinical trials for pain relief are primarily improved health-related quality of life and functionality, rather than nociceptive thresholds.^{55,84} However, preclinical pain research has historically relied on mechanical and thermal hypersensitivity as a primary outcome in rodent pain models, which represents only one component of human chronic pain. To address this translational gap, substantial efforts have been directed towards the assessment of voluntary behaviors and quality of life measures in rodent pain models that better reflect how pain impacts the lives of patients.^{2,43,85}

Clinical studies demonstrate that chronic pain reduces quality of life in part by impairing mobility as well as physical and social activities, and increasing anxiety symptoms.^{20,25,27,30,53,77,90} Accordingly, one goal of preclinical pain researchers is to develop and utilize measures of pain-related suppressed and evoked behaviors.⁵² However, preclinical studies evaluating the effects of inflammation and nerve injury on rodent gait, locomotion, social interaction, and anxiety-like behavior have yielded conflicting results. In some cases, inflammation and nerve injury have been shown to alter gait^{8,14,46,59–61}, suppress general locomotion and voluntary wheel running^{12,24,31,42,60,61,82,83}, reduce social interactions^{56,64}, and/or induce anxiety-like behavior^{17,36,50,62,70,92}; however, in other cases, these behaviors were unchanged by persistent pain.^{9,23,47,73,87} These conflicting results may be the product of differences in study design with

respect to species, injury model, behavioral paradigms, etc. Thus, whether these endpoints are valid measures of rodent pain-related behavior remains unresolved.

In the present study, we systematically evaluated voluntary wheel running, locomotion, gait, social interaction, and anxiety-like behavior in two commonly used mouse models of persistent pain: Complete Freund's Adjuvant-induced inflammation and the spared nerve injury model of neuropathic pain. When appropriate, we tested for correlations between changes in voluntary behavior and mechanical hypersensitivity, a widely used stimulus-evoked/reflexive endpoint. Further, to determine whether changes in voluntary behaviors were pain-related, we tested if they could be reversed with an analgesic. We utilized the angiotensin II type 2 receptor antagonist PD123319, a promising candidate analgesic that has been shown to be effective in a variety of rodent peripheral nerve injury models^{49,74,79,80}, and is related to the compound EMA401 that has shown efficacy in a phase II clinical trial for neuropathic pain.⁶⁶

2.3 Materials and Methods

Experimental Animals

Animals were cared for in compliance with the National Institutes of Health guidelines and approved by the Animal Studies Committee of Washington University in St. Louis (Protocol Numbers 20130147, 20160097). Experiments were predominantly conducted on adult C57BL/6J male mice bred in house using breeding pairs from Jackson Labs. (Bar Harbor, Maine). One cohort of adult experimental animals was obtained directly from Jackson Labs and allowed to acclimate to our animal housing facility for at least 1 week before initiating behavioral testing. Mice were housed with up to 4 cagemates in the animal facility under a 12-hour (hr) light/dark cycle (6AM-6PM) and provided food and water *ad libitum*. Cages were lined with corncob bedding. Behavioral experiments began when mice were 7-9 weeks of age. Throughout experiments, animals were regularly monitored for general health and weighed weekly. At the conclusion of each study, mice were euthanized using a rodent ketamine euthanasia cocktail.

Experimental Models of Pain

Throughout all surgical procedures, mice were anesthetized with 2% isoflurane. Intraplantar Complete Freund's Adjuvant (CFA) was used as a model of persistent inflammatory pain. Mice received a single bilateral intraplantar hindpaw injection of 20 μ L undiluted CFA (1 mg/mL; Sigma, St. Louis, MO). Control mice were similarly injected bilaterally with 20 μ L of 0.9% sterile saline. Behavioral testing began as soon as 4 hr post injection.

Unilateral spared nerve injury (SNI) was performed as described previously.^{16,67,73} Akron lidocaine hydrochloride jelly (2%; Vessel Medical, Greenville, SC) was applied topically to the incision site. The three branches of the sciatic nerve were exposed by separating the biceps femoris muscle, and the common peroneal and tibial branches were ligated with silk suture and cut distal to the ligation, taking care not to manipulate the sural nerve. Sham operation comprised a skin incision over the biceps femoris muscle. For all operations, the skin incision

was closed with staples that were removed on postoperative day (POD) 7, once wounds had healed. Allowing time for post-operative recovery, behavioral testing began no sooner than POD 4. Upon completion of behavioral studies, SNI mice were dissected to confirm that the sural nerve was not included in or damaged by the ligation surgery. If the sural nerve was not intact, animals were excluded from the study (4% of SNI mice).

CFA-Induced Paw Edema

Paw thickness (in millimeters) was measured using 150 mm stainless dial calipers (Chicago Brand, Medford, OR). Paw thickness was measured at baseline, as well as post-intraplantar injection of either CFA or saline.

Behavioral Studies

For each experimental cohort, animal groups were randomized and the experimenter was blind to injury and/or drug treatment groups until the completion of data analysis. Unless otherwise specified, prior to behavioral testing, mice were acclimated to the testing apparatus (von Frey) or the testing room within their homecages (voluntary wheel running, open field, rotarod) for 1-2 hr with low-level white noise. In cases where both baseline and post-injury measurements were made, data for each mouse is reported as normalized to baseline (von Frey, voluntary wheel running, gait analysis). Experiments were performed during the light cycle between 8 AM and 5 PM.

Sample sizes for each behavioral endpoint were modeled off of group sizes required to observe physiologically significant behavioral effects in previously published rodent studies of voluntary behaviors.^{60,61,76} Where correlation analyses were planned, additional animals were tested to meet the adequate sample size to make comparisons between behavioral endpoints. Cohorts were comprised of 10-15 mice from a single source (either in house or Jackson Labs).

At least two cohorts of animals were tested for each behavioral endpoint to obtain the appropriate sample size and evaluate whether injury- or drug-induced changes produced similar effects across cohorts. All CFA cohorts were bred in house and all but one SNI cohort (evaluated in von Frey, voluntary wheel running, and the social interaction assay) was bred in house. Similar SNI-induced behavioral effects were observed across cohorts regardless of the animal source.

Mechanical Sensitivity (von Frey)

Mechanical sensitivity was measured using the up-down method of the von Frey test.⁷ Prior to baseline behavioral testing, mice were habituated to the elevated mesh grid in Plexiglass boxes for 2 hr/day for 2 days. On testing days, calibrated filaments (North Coast Medical Inc., Gilroy, CA) were applied to the plantar surface of the hindpaw. In SNI studies, baseline and post-operative mechanical sensitivity was measured on the lateral aspect of the hindpaw.¹⁸ In all studies, 3 trials were conducted on each paw, with at least 5 min between testing opposite paws, and at least 10 min between testing the same paw. Mechanical withdrawal thresholds of each paw were calculated by averaging values obtained across trials. We established a von Frey threshold inclusion criterion to ensure that an adequate dynamic range existed for detecting mechanical hypersensitivity following injury. In order for an animal to be tested post-injury, baseline thresholds had to be greater than or equal to 0.20 g. Of the total number of mice that underwent baseline testing, 98% of CFA mice (tested on the center of the paw) and 78% of SNI mice (tested on the lateral aspect of the paw) met the inclusion criterion.

Voluntary Wheel Running

Voluntary wheel running was quantified using wireless low-profile running wheels (Med Associates, Fairfax, VT). To reduce environmental novelty, mice were first acclimated to

individual cages with locked running wheels for 2 hr/day for 2 days. Running wheels were then unlocked for baseline behavioral testing. Distance travelled was recorded for 2 hr without the experimenter in the room. Mice were classified as non-runners if they ran less than 200 m during the baseline session and were not tested for voluntary wheel running post-injury. Of all animals that underwent baseline testing, 10% were non-runners. Wheel running experiments were conducted during the light cycle between 11 AM and 1 PM.

Social Interaction

Social interaction was measured using a social approach assay described previously.⁷⁶ Room lighting was ~200 lux. The black, plastic testing arena (52.5 L X 25.5 W X 25.5 H cm), the bottom of which was covered with corncob bedding, consisted of two-chambers, each containing an inverted metal, mesh pencil cup. One pencil cup was arbitrarily designated to lie within the social zone. The social zone was defined as a circle equal to twice the diameter of the pencil cup. Prior to testing, mice were acclimated to a silent room in their homecages for 1 hr. In the baseline trial, the test mouse moved freely within the arena for 10 min once the experimenter left the room. EthoVision XT video tracking software (Noldus, Cincinnati, OH) was used to monitor animal movement and quantify time spent within the social zone. Following the baseline trial, the test mouse was returned to its homecage for 30 min. An unfamiliar, age-, sex-, and strain-matched conspecific stimulus mouse was then placed beneath the social zone pencil cup. In the social trial, the test animal was reintroduced into the arena for 10 min and the time spent in the social zone was recorded. Test mice could see, hear, and smell, but not physically interact with the stimulus mouse within the pencil cup. Social interaction scores were determined by dividing the amount of time the test mouse spent in the social zone during the social trial by the amount of time the test mouse spent in the social zone during the baseline trial.

Locomotor Activity and Anxiety-Like Behavior (Open Field)

Locomotor activity was quantified in an open field chamber equipped with a Versamax Animal Activity Monitoring System (AccuScan Instruments Inc., Columbus, OH) under normal laboratory light (~770 lux). Following acclimation, mice were individually placed into the center of the open field and allowed to explore the chamber for 1 hr once the experimenter left the room. Total distance traveled and time spent moving were calculated for the 42 L X 42 W X 20 H cm chamber. Anxiety-like behavior was evaluated by quantifying the percentage of time spent in the center zone of the open field arena over the 1 hr testing period. The center zone was defined as a square comprising 40% of the arena. As open field activity is largely driven by exploratory behavior of a novel environment¹⁵, mice were only tested in the open field once. Thus, open field activity across multiple post-CFA timepoints was acquired from separate cohorts of mice.

Gross Motor Function (Rotarod)

Motor coordination and balance was determined using an accelerating rotarod (Ugo Basile, Varese, Italy). First, a training session involving 120 s on a non-accelerating rotarod was conducted, followed by 1 hr of rest with mice in their homecages. Then 5 consecutive trials with 5 min rest intervals between trials were performed. Latency to fall as the rotarod accelerated from 4 to 40 rpm in 30 s increments over 5 min was recorded.

Catwalk Automated Gait Analysis

Gait analysis was performed using the Catwalk XT 10.5 system (Noldus, Cincinnati, OH). Briefly, the Catwalk XT system consists of an elevated, enclosed glass platform (130 L X 7 W cm) and a high-speed camera (GEViCAM GP-2360C). Green light is internally reflected into the glass platform and light is emitted downward only when pressure is placed upon the glass

(i.e. by an animal's paw). The high-speed camera detects emitted light intensity per pixel, and the accompanying Catwalk XT software acquires and analyzes gait parameters. Gait analysis data were acquired using the following experimental settings: camera gain: 13-15 dB, green light intensity threshold: 0.12, run duration: 0.5-5.0 s, run maximum variation: 60%. In some cases, the ipsilateral hindpaw of SNI mice was not detectable using these experimental settings and these animals were ineligible for analysis.

The day prior to baseline behavioral testing, mice were acclimated to the Catwalk platform over two 15 min sessions separated by 30 min. On testing days, mice were habituated to the testing room for at least 30 min and behavioral testing was conducted in a completely dark, silent room. Mice voluntarily traversed the enclosed glass platform. At each testing timepoint, 4 compliant runs were obtained and averaged per mouse. Postoperative Catwalk testing on SNI animals began after surgical staples were removed on POD 7, as the staples could have subtle effects on gait.

Parameters obtained and reported from Catwalk gait analysis include:

Paw pressure (max contact, mean intensity): Average print intensity (a.u.) when the paw is making maximum contact with the glass.

Run speed: Average body speed (cm/s)

Stance phase: Duration (s) that paw is in contact with the glass platform

Swing phase: Duration (s) that paw is *not* in contact with the glass platform

Step duration: Sum (s) of stance and swing phase

Fraction stance phase: Stance phase divided by step duration

Fraction swing phase: Swing phase divided by step duration

Maximum contact area: Maximum area (cm²) of the paw that contacts the glass platform

Drugs and Drug Administration

PD123319 ditrifluoroacetate (Tocris, Minneapolis, MN), also known as EMA200, was dissolved in 0.9% sterile saline and injected at 10 mg/kg, i.p. Vehicle controls were injected with an equal volume of saline. Notably, previous studies demonstrate that systemic administration of 10 mg/kg PD123319 attenuates hypersensitivity without impairing motor coordination in the rotarod test.^{58,79} The effects of PD123319 on mechanical hypersensitivity and Catwalk hindpaw pressure were evaluated over 3 testing sessions per behavioral assay: pre-drug, drug, and post-drug sessions. Each testing session was separated by 24-48 hr. All experiments evaluating the effects of PD123319 were performed between POD 7 and POD 41, when SNI mice were known to be hypersensitive. Mechanical hypersensitivity testing sessions were performed on either POD 12-16 or 26-30 and results from these timepoints were pooled together. Catwalk hindpaw pressure testing sessions were performed on POD 9-12. Behavioral testing took place 1.5-2.5 hr post injection, the time frame at which peak analgesia with respect to mechanical hypersensitivity has been reported.^{74,79}

Statistical Analyses

All data were analyzed using Excel (Microsoft, Redmond, WA) and Prism (GraphPad Software, Inc., La Jolla, CA) and are presented as mean \pm SEM. Significance was defined as $p < 0.05$ a priori. Post-injury withdrawal thresholds, running distances, Catwalk gait parameters, and paw thickness are normalized to baseline values measured prior to inflammation or nerve injury. Figure legends indicate the group size and statistical test for each experiment. Briefly, data comparing the effect of CFA or SNI on paw thickness, body weight, and behavior over time were analyzed with a Student's t-Test and Holm-Sidak correction for multiple comparisons, when appropriate. The effects of PD123319 on SNI-induced behavioral changes were analyzed using a two-way repeated-measures (RM) ANOVA, post-hoc Student's t-Tests, and Sidak

correction for multiple comparisons. Pearson correlation coefficients were determined to evaluate the relationship between CFA- or SNI-induced mechanical hypersensitivity and changes in voluntary behaviors.

2.4 Results

CFA induced paw edema and mechanical hypersensitivity

To determine the effects of persistent inflammation on rodent stimulus-evoked and voluntary behaviors, we utilized the intraplantar Complete Freund's Adjuvant (CFA) model of inflammatory pain.^{28,63} Following bilateral intraplantar injections of CFA, we observed significant thickening of the hindpaws that persisted for at least 14 days relative to saline-injected controls (Fig. 1A). Intraplantar CFA is known to produce robust hypersensitivity that persists for at least 1-2 weeks.^{12,28,63,78} Similarly, we observed a significant reduction in hindpaw mechanical withdrawal thresholds in CFA mice compared to saline mice (Fig. 1B). Mechanical hypersensitivity was detectable on post injection day (PID) 0 (~4 hr after the injection) through PID 9. Interestingly, CFA-induced mechanical hypersensitivity recovered by PID 14 despite persistent paw edema.

CFA suppressed voluntary wheel running

To determine whether persistent inflammation alters voluntary behaviors, we tested mice between PID 0 and 7, when CFA-induced mechanical hypersensitivity was most robust. We first assessed whether inflammation suppressed voluntary wheel running. In order to decrease the likelihood of exercise-induced analgesia^{11,33}, wheel running distances were quantified every other day beginning 1 day after injection. We initially tested whether unilateral intraplantar CFA suppressed voluntary wheel running. In alignment with a previous report¹², unilateral CFA did not affect wheel running activity compared to saline (data not shown). We next tested whether bilateral CFA affected voluntary wheel running. Relative to saline, bilateral CFA produced a significant reduction in voluntary wheel running on PID 1 (Fig. 1C). Wheel running of CFA mice began to recover on PID 3 and reached that of saline mice by PID 7, despite continued CFA-induced mechanical hypersensitivity through PID 9. The time course of recovery from CFA-

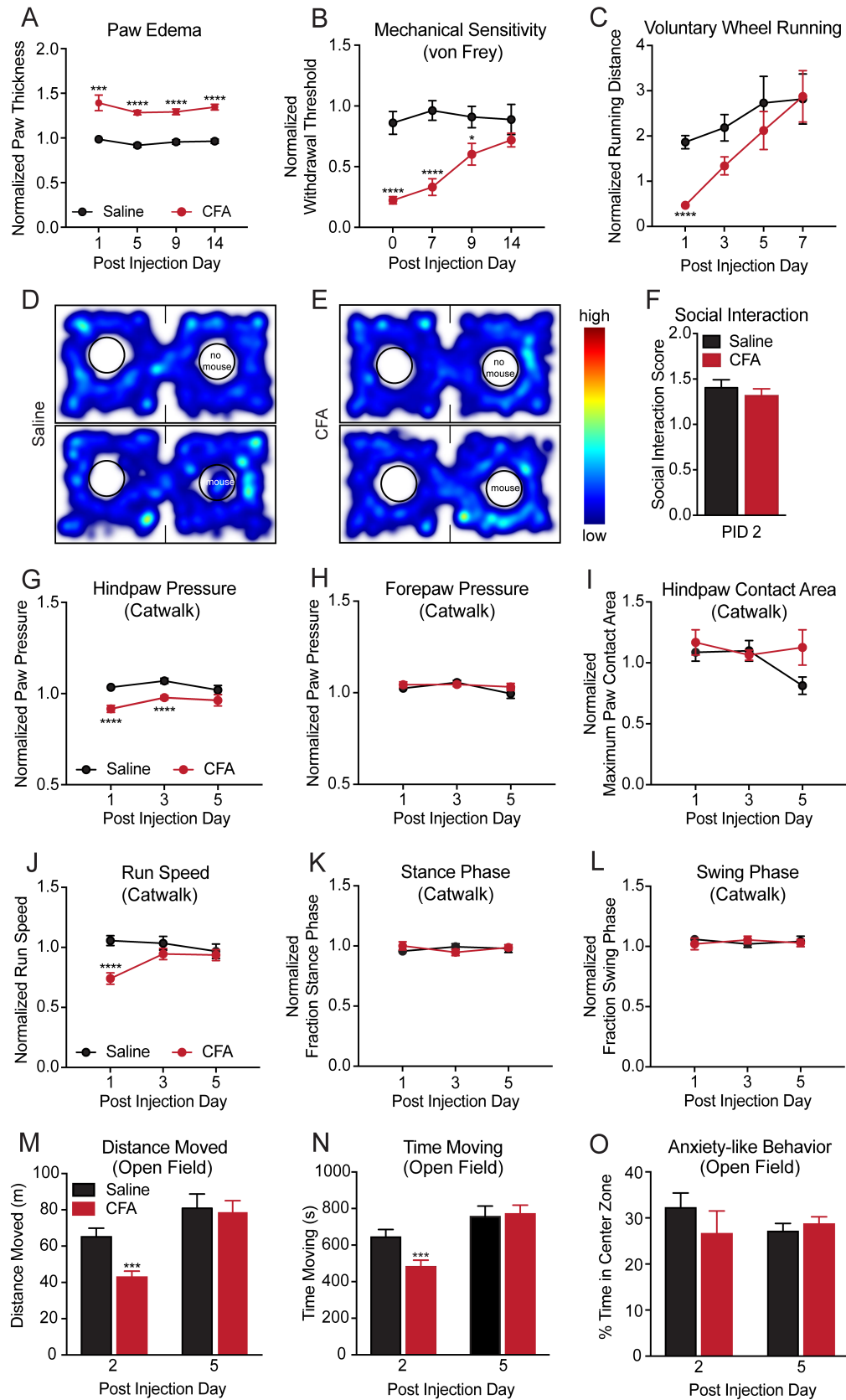


Figure 1. Bilateral CFA suppressed locomotion and altered gait, but did not affect social interaction or anxiety-like behavior. (A) Bilateral intraplantar injection of Complete Freund's Adjuvant (CFA) produced significant paw edema that persisted for at least 14 days compared to injection of saline (n=10-17/group). (B) CFA mice displayed a significant reduction in mechanical hindpaw withdrawal thresholds as soon as 4 hr post injection (day 0) through post injection day (PID) 9 compared to saline mice (n=10-17/group). (C) Voluntary running was significantly reduced by CFA on PID 1 compared to saline (n=11-23/group). Representative heatmaps from the social interaction test of (D) saline and (E) CFA mice in the absence (top) and presence (bottom) of an unfamiliar conspecific. Scale bar indicates total time spent in each arena location. Presence within circles reflects time spent atop the pencil cups. (F) Social interaction scores were equivalent between CFA and saline mice on PID 2 (n=12/group). (G, H) Catwalk gait analysis demonstrated that CFA significantly decreased hindpaw pressure though PID 3 compared to saline, yet no changes in forepaw pressure were observed (n=12/group). (I) Despite the paw edema of CFA mice, hindpaw contact area with the Catwalk platform was equivalent between CFA and saline mice. (J, K, L) CFA caused a significant reduction in run speed across the Catwalk platform on PID 1; however no differences in stance phase or swing phase were observed between CFA and saline mice. (M, N) CFA significantly reduced distance moved and time spent moving in an open field compared to saline on PID 2 (n=11-13). (O) CFA mice did not display anxiety-like behavior in an open field compared to saline mice at either timepoint tested. Data are presented as mean \pm SEM. Student's t-Test, Holm-Sidak correction for multiple comparisons, when appropriate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, as compared with saline mice.

induced suppression of voluntary wheel running closely resembles that reported by others^{12,24,31,60}, suggesting that the effects of CFA on voluntary wheel running are consistent and reproducible. As bilateral CFA administration was required to observe suppression of wheel running, all subsequent investigations of the effects of inflammation on voluntary behavior were conducted using bilateral CFA injections.

CFA did not affect social interactions

To determine whether persistent inflammation decreased social interaction, we used a behavioral model of social approach.^{75,76} Social interaction scores of CFA mice with an unfamiliar age-, sex-, and strain-matched conspecific were equivalent to saline mice on PID 2 (Fig. 1D-F). Our findings support previous work demonstrating that acutely after injection, CFA does not affect social interactions of mice that are hypersensitive to mechanical stimuli.³⁶

CFA induced gait alterations

To evaluate whether bilateral CFA elicited static and/or dynamic gait deficits, we utilized the Catwalk XT gait analysis system. With respect to static gait parameters, compared to saline,

CFA significantly reduced hindpaw pressure through PID 3 (Fig. 1G) without altering forepaw pressure (Fig. 1H). Paw pressure was determined at the point of maximum contact of the paw with the Catwalk platform. Therefore, we evaluated whether CFA-induced paw edema could be affecting hindpaw pressure by increasing maximum hindpaw contact area. However, compared to saline, CFA administration did not significantly alter hindpaw maximum contact area (Fig. 1I).

With respect to dynamic gait parameters, CFA significantly decreased run speed on PID 1 compared to saline (Fig. 1J). Many gait parameters are dependent on run speed.³ Therefore, stance phase and swing phase were calculated as a fraction of the total step duration, the combined duration of the stance and swing phases. No changes in stance phase or swing phase were observed following CFA (Fig. 1K, 1L). In contrast to these findings, previous reports of rodent gait analysis following unilateral intraplantar CFA demonstrate a broader effect of inflammation on gait. For instance, reduced stance phase and prolonged swing phase of the injected hindlimb have been proposed to reflect pain avoidance behavior.^{14,59,60} In our studies, however, bilateral injury likely precluded the ability to observe possible inflammation-induced avoidance behaviors in hindpaw gait parameters. Collectively, our findings demonstrate that bilateral CFA produces transient, yet significant changes in a subset of static and dynamic gait parameters.

CFA induced changes in open field behavior

To determine whether persistent inflammation affected locomotor and/or anxiety-like behavior of mice, open field activity was evaluated. On PID 2, CFA mice displayed significantly decreased distance moved and time spent moving within the open field compared to saline mice (Fig. 1M, N). These locomotor deficits were no longer present in mice tested on PID 5. Similarly, Refsgaard et al. report that open field locomotor behavior is unchanged on PID 4 following unilateral intraplantar CFA.⁶² In the open field test, anxiety-like behavior is expressed as a

reduction in the proportion of time spent in the center zone of the open field compared to a control condition. In our studies, time spent in the center of the open field was comparable between CFA and saline mice on all testing days (Fig. 1O). These results suggest that mice do not exhibit anxiety-like behavior in the open field after inflammation, and are supported by previous findings.^{36,87}

SNI induced mechanical hypersensitivity

We next tested whether nerve injury also caused changes in voluntary behavior. To determine the effects of nerve injury on rodent stimulus-evoked and voluntary behaviors, we utilized the spared nerve injury (SNI) model of neuropathic pain. SNI has been shown to produce prolonged mechanical hypersensitivity in the lateral aspect of the hindpaw.^{16,73,87} We similarly observed a significant reduction in hindpaw mechanical withdrawal thresholds of SNI mice compared to sham-operated mice by postoperative day (POD) 7, which persisted through at least POD 40 (Fig. 2A).

SNI did not suppress voluntary wheel running or social interaction

To test whether nerve injury suppressed voluntary wheel running, running distances were quantified at multiple postoperative timepoints between POD 5 and POD 41. At each timepoint, SNI mice and sham mice displayed equivalent wheel running activity (Fig. 2B). These findings were obtained during the light cycle and are supported by our previous observations that SNI mice ran equivalent distances to uninjured mice when given wheel access for either 2 or 12 hr per night, the time at which mice are most active.⁷³

Similarly, we tested whether nerve injury suppressed social interaction with an unfamiliar age-, sex-, and strain-matched conspecific. We observed that social interaction scores were unchanged in SNI mice relative to sham mice on POD 8 or 14 (Fig. 2C-E).

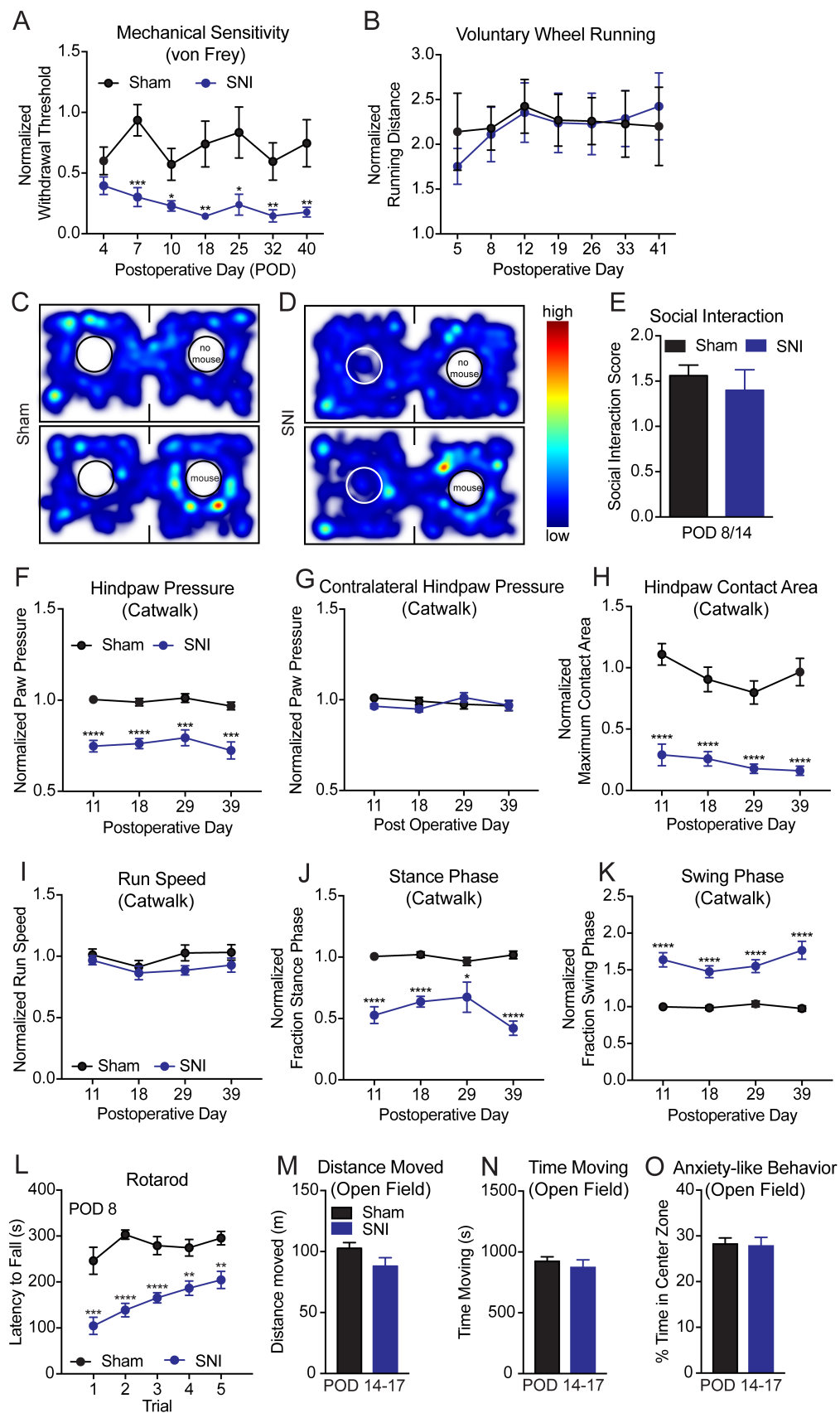


Figure 2. SNI produced gait alterations, but did not influence locomotion, social interaction, or anxiety-like behavior. (A) Spared nerve injury (SNI) caused a significant reduction in hindpaw mechanical withdrawal thresholds compared to sham operation on postoperative days (POD) 7-40 (n=10-17/group). (B) SNI and sham mice exhibited equivalent wheel running activity at each testing timepoint between POD 5 and POD 41 (n=12-18/group). Representative heatmaps from the social interaction test of (C) sham and (D) SNI mice in the absence (top) and presence (bottom) of an unfamiliar conspecific. Presence within circles reflects time spent atop the pencil cups. Scale bar indicates total time spent in each arena location. (E) Social interaction scores were equivalent between sham and SNI mice on POD 8/14 (n=15/group). (F, G) Catwalk gait analysis demonstrated a significant reduction in ipsilateral hindpaw pressure, but no change in contralateral hindpaw pressure of SNI mice relative to sham mice on POD 11 through POD 39 (n=10-23/group). (H) SNI significantly decreased hindpaw contact area compared to sham surgery. (I) Catwalk run speed did not differ between SNI and sham mice. (J, K) SNI mice had a significantly shorter stance phase and significantly longer swing phase relative to sham mice on POD 11 through POD 39. (L) SNI mice were impaired in the rotarod test, and had a significantly shorter latency to fall compared to sham mice across all trials on POD 8 (n=10-12/group). (M, N) SNI did not affect distance moved or time spent moving in the open field test on POD 14-17 (n=12-16). (O) SNI mice did not exhibit anxiety-like behavior in the open field test compared to sham mice on POD 14-17. Data are presented as mean \pm SEM. Student's t-Test, Holm-Sidak correction for multiple comparisons, when appropriate. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, as compared with sham mice.

Interestingly, previous studies show SNI-induced decreases in rodent social interaction at postoperative timepoints more acute (POD 5)^{64,94}, but not long-term (POD 40)⁸⁷, than those we tested. Taken together, our results demonstrate that despite ongoing mechanical hypersensitivity, neither voluntary wheel running nor social interaction were suppressed by nerve injury.

SNI induced gait and motor deficits

Using the Catwalk gait analysis system, we tested whether nerve injury altered gait. Significant changes in both static and dynamic gait parameters were observed after SNI from POD 11 to 39. Analysis of static gait parameters revealed that compared to sham-operated mice, ipsilateral hindpaw pressure of SNI mice was significantly decreased (Fig. 2F). However, no changes in contralateral hindpaw pressure were observed (Fig. 2G). As with other rodent sciatic nerve injury models^{68,93}, SNI gave rise to cupping of the hindpaw, highlighted by a significant reduction in hindpaw maximum contact area compared to sham-operated mice (Fig. 2H). Analysis of dynamic gait parameters demonstrated that the run speed of SNI mice was comparable to sham mice (Fig. 2I). However, SNI mice displayed a significantly shorter stance

phase (Fig. 2J), as well as significantly longer swing phase (Fig. 2K) relative to sham controls. Interestingly, in contrast to bilateral CFA, all static and dynamic gait deficits following SNI persisted as long as mechanical hypersensitivity, through at least POD 39.

Multiple studies have demonstrated gait deficits following nerve injury in rodents.^{8,46,59,61} However, whether these effects represent a pain-avoidance behavior or simply a motor deficit is uncertain.⁸⁵ To address this, we tested gross motor coordination of nerve-injured mice using the accelerating rotarod test on POD 8. Indeed, SNI mice displayed a significant motor impairment across all test trials. SNI mice had a significantly shorter latency to fall off the rotarod than sham mice (Fig. 2L). Urban et al. also observed SNI-induced motor impairment in the rotarod test.⁸⁷ Thus, SNI-induced gait deficits may be the product of gross motor deficits.

SNI did not induce changes in open field behavior

To determine whether nerve injury suppressed locomotor behavior or induced anxiety-like behavior, we evaluated open field activity on POD 14-17. SNI mice did not differ from sham mice with respect to distance moved (Fig. 2M) or time spent moving (Fig. 2N) in the open field test. Our findings support those of existing studies demonstrating that gross locomotion of mice is unchanged after SNI.^{9,87,94} Lastly, percent time spent in the center zone of the open field was equivalent between SNI mice and sham mice (Fig. 2O), suggesting that at this timepoint, nerve-injured mice were not in a general anxiety-like state, as supported by previous findings.⁸⁷

Summary of CFA- and SNI-induced changes in voluntary behavior and animal wellbeing

We demonstrated that inflammation and nerve injury differentially affect voluntary behaviors (Table 1). In addition to causing mechanical hypersensitivity, bilateral intraplantar injection of CFA transiently suppressed voluntary wheel running, decreased locomotor activity, and altered static and dynamic gait parameters. However, neither social interactions nor

anxiety-like behavior were affected by CFA (Fig. 1). Interestingly, all inflammation-induced changes in voluntary behaviors resolved by PID 5, whereas inflammation-induced mechanical hypersensitivity persisted through PID 9.

Table 1. Inflammation and nerve injury differentially affect voluntary behaviors while mice display mechanical hypersensitivity

Behavioral Endpoint	CFA, bilateral	SNI, unilateral
Mechanical withdrawal threshold	↓	↓
Wheel running	↓	no Δ
Gait	Δ	Δ
Social interaction	no Δ	no Δ
Open field-locomotion	↓	no Δ
Open field-anxiety	no Δ	no Δ

↓ indicates significantly decreased behavior; Δ indicates significant behavioral changes; no Δ indicates absence of significant behavioral changes; CFA, Complete Freund's Adjuvant; SNI, spared nerve injury.

In contrast, despite robust and persistent mechanical hypersensitivity, SNI did not suppress voluntary wheel running, social interactions, or locomotor activity. Further, SNI did not induce general anxiety-like behavior in the open field. SNI did produce changes in both static and dynamic gait parameters that, like mechanical hypersensitivity, persisted the entire length of our study, through POD 39 (Fig. 2). However, it is possible that these gait alterations are in part the product of a general motor deficit, as suggested by impaired rotarod performance of SNI mice.

In addition to evaluating voluntary behaviors following injury, we also monitored body weight as a measure of general animal health.⁶ CFA mice had body weights equivalent to saline mice through PID 14 (Fig. 3A). Likewise, body weights of SNI mice were equivalent to sham mice through postoperative week 6 (Fig. 3B). In summary, despite ongoing mechanical

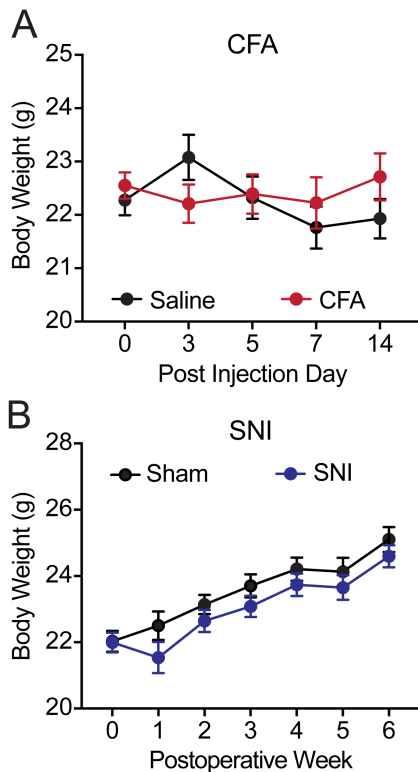


Figure 3. Neither CFA nor SNI affected mouse body weight. (A) Body weight of Complete Freund's Adjuvant (CFA) mice was equivalent to saline mice from PID 0 through PID 14 (n=13-42/group). (B) Body weights of spared nerve injury (SNI) and sham mice were equivalent between postoperative weeks 0 and 6 (n=23-38/group). Data are presented as mean \pm SEM. Student's t-Test, Holm-Sidak correction for multiple comparisons.

hypersensitivity, inflammation and nerve injury do not negatively impact overall animal health, and have primarily short-lived effects on voluntary behaviors.

CFA- and SNI-induced changes in voluntary behavior did not correlate with mechanical hypersensitivity

When changes in voluntary behavior were observed after inflammation or nerve injury, we performed correlation analyses to test whether the degree of change in voluntary behavior correlated to the degree of change in the mechanical withdrawal threshold for a given animal. Following bilateral CFA, attenuated hindpaw mechanical withdrawal thresholds on PID 0 were not significantly correlated with decreased voluntary wheel running on PID 1 (Fig. 4A, $r(10)=-0.349$, $p=0.267$), as has been reported previously in rats.²⁴ It has been suggested that hindpaw pressure measured via the Catwalk gait analysis system provides an objective readout of mechanical

hypersensitivity. For instance, in some cases, a correlation has been reported between mechanical hypersensitivity and decreased hindpaw pressure following inflammation or nerve injury in rodents.^{21,46,89} However, we found no correlation between CFA-induced mechanical hypersensitivity on PID 0 and reduced Catwalk hindpaw pressure on PID 1 (Fig. 4B, $r(21)=0.248$, $p=0.255$). Likewise, following SNI, attenuated hindpaw withdrawal thresholds on POD 7 did not correlate with decreased Catwalk hindpaw pressure measured on POD 11 (Fig. 4C, $r(17)=-0.0077$, $p=0.9750$). Taken together, our results demonstrate that neither

inflammation- nor nerve injury-induced changes in voluntary behavior correlate to mechanical hypersensitivity. Thus, while each endpoint requires mice to ambulate on the injured hindpaw(s), voluntary wheel running and hindpaw pressure are not simply alternative measures of mechanical hypersensitivity.

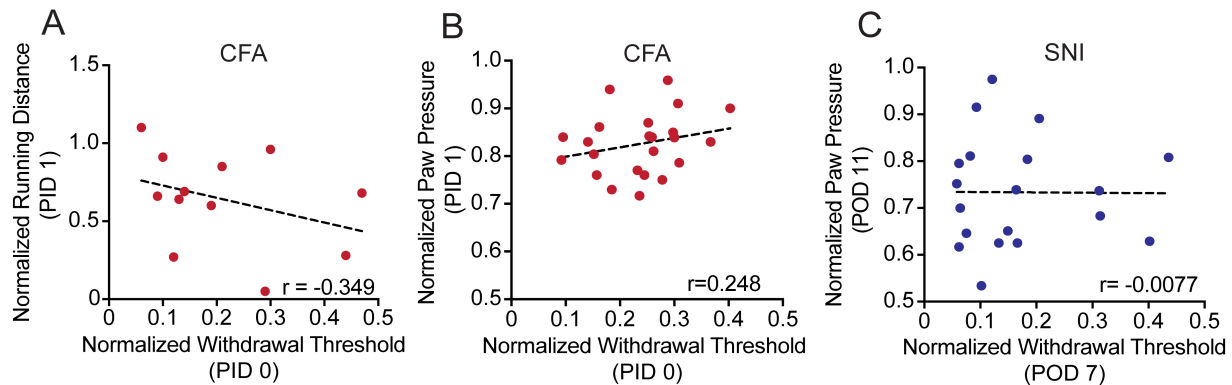


Figure 4. CFA- and SNI-induced changes in voluntary behavior did not correlate with mechanical hypersensitivity. (A) Following bilateral Complete Freund's Adjuvant (CFA), reductions in hindpaw withdrawal thresholds on post injection day (PID) 0 did not correlate with decreased voluntary wheel running distances ($n=12$, $r(10) = -0.349$, $p=0.267$) or (B) reduced Catwalk hindpaw pressure ($n=23$, $r(21) = 0.248$, $p=0.255$) measured on PID 1. (C) Attenuated hindpaw mechanical withdrawal thresholds of spared nerve injury (SNI) mice on postoperative day (POD) 7 did not correlate with reduced Catwalk hindpaw pressure on POD 11 ($n=19$, $r(17) = -0.0077$, $p=0.9750$). Each data point represents one individual mouse. Correlation analyses were performed to calculate the Pearson correlation coefficient (r).

SNI-induced mechanical hypersensitivity, but not reduced hindpaw pressure, was reversed by an analgesic

Lastly, we tested if the angiotensin II type 2 receptor antagonist PD123319 could reverse SNI-induced gait deficits to determine whether these were indeed pain-related changes. We were particularly interested in the effects of PD123319 on SNI-induced reductions in hindpaw pressure, which has been recommended as an objective measure of mechanical hypersensitivity.^{14,21,61,89} Administration of PD123319 (10 mg/kg, i.p.), but not saline, significantly increased mechanical withdrawal thresholds of SNI mice (Fig. 5A, two-way RM ANOVA, Sidak correction: $F(2,42) = 3.816$, $p=0.03$ for drug group x test session interaction). This interaction was

driven by significantly increased mechanical withdrawal thresholds of SNI mice treated with PD123319 compared to those treated with saline on the day of drug administration (Post-hoc Student's t-Test: $t=3.018$, $p=0.0110$), and significantly increased mechanical withdrawal thresholds within the PD123319 group on the drug session compared to pre-drug and post-drug sessions (Post-hoc Student's t-Tests: for pre-drug compared to drug, $t=4.672$, $p<0.0001$; for post-drug compared to drug, $t=4.984$, $p<0.0001$). In contrast, compared to saline, administration of PD123319 did not significantly alter SNI-induced decreases in Catwalk hindpaw pressure, and no differences were observed within the PD123319 group across testing sessions (Fig. 5B). SNI-induced changes in hindpaw contact area, stance phase, and swing phase were similarly unaffected by PD123319 (data not shown). Furthermore, compared to pre-drug and post-drug sessions, PD123319 did not alter

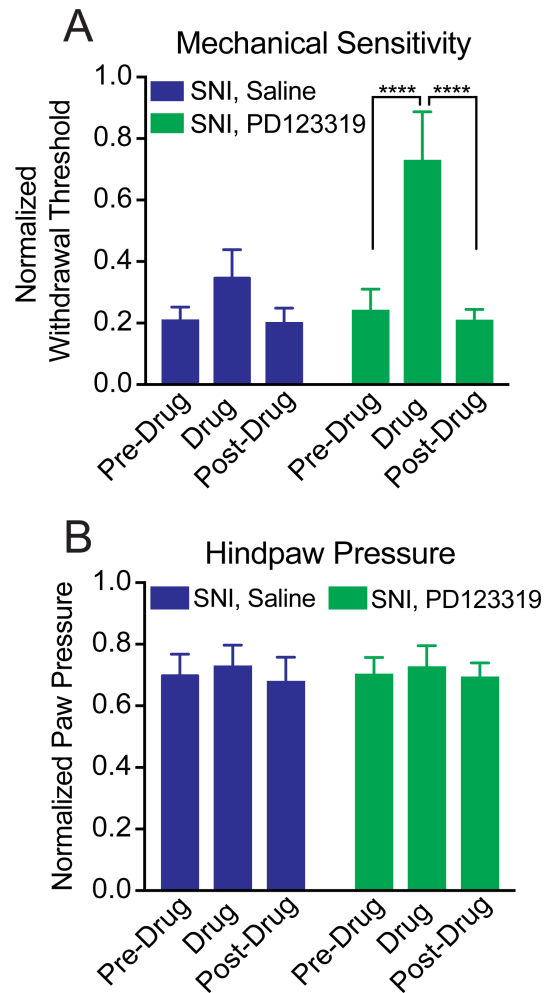


Figure 5. The analgesic PD123319 reversed SNI-induced mechanical hypersensitivity, but not SNI-induced decreases in Catwalk hindpaw pressure. (A). Systemic administration (10 mg/kg, i.p.) of the angiotensin II type 2 receptor antagonist PD123319, but not saline, significantly increased mechanical withdrawal thresholds of spared nerve injury (SNI) mice on postoperative days (POD) 12-15 or 26-30. Data from these timepoints were pooled together. ($n=11-12/\text{group}$, two-way RM ANOVA, Sidak Correction: $F(2,42)=3.816$, $p=0.03$ for drug group \times test session interaction). Mechanical withdrawal thresholds of SNI, PD123319 were significantly greater than SNI, saline mice following drug administration (Post-hoc Student's t-Test: $t=3.018$, $p=0.0110$). Within the SNI, PD123319 group, drug administration significantly increased mechanical withdrawal thresholds compared to pre-drug and post-drug sessions. (Post-hoc Student's t-Tests: for pre-drug compared to drug, $t=4.672$, $****p<0.0001$; for post-drug compared to drug, $t=4.984$, $****p<0.0001$). (B) Compared to pre-drug and post-drug sessions, or administration of saline, PD123319 did not change Catwalk hindpaw pressure of SNI mice on POD 9-12 ($n=5-8/\text{group}$). Data are presented as mean \pm SEM.

mechanical withdrawal thresholds or hindpaw pressure of the contralateral, uninjured hindpaw of SNI mice (data not shown). In summary, PD123319 significantly reversed SNI-induced mechanical hypersensitivity, but had no effect on SNI-induced reductions in Catwalk hindpaw pressure. Our findings contribute to the growing literature from both rats and mice demonstrating that nerve-injury induced gait deficits are not reversed by analgesics.^{46,59}

Although we also observed CFA-induced decreases in voluntary wheel running and Catwalk hindpaw pressure, we did not test whether these changes in voluntary behavior could be reversed by known analgesics. Cobos et al. have thoroughly demonstrated that non-steroidal anti-inflammatory drugs and morphine prevent inflammation-induced suppression of voluntary wheel running.¹² While to our knowledge prevention or reversal of gait deficits resulting from intraplantar CFA have not yet been demonstrated, we observed a relatively small decrease in hindpaw pressure in CFA mice (8.6% decrease from baseline, Fig. 1G), which provides little dynamic range for reversal by an analgesic.

2.5 Discussion

In response to the widely critiqued translational gap between preclinical and clinical measures of pain^{2,10,13,37,43,45,65,72,85,88}, we evaluated whether voluntary behaviors are interrupted in rodent models of persistent pain. Specifically, we tested for inflammation- and nerve injury-induced changes in voluntary wheel running, locomotion, gait, social interaction, and general anxiety-like behavior. There is currently conflicting evidence regarding the relevance of these endpoints as pain-related behaviors. However, in order to establish a new, reliable rodent pain-related behavior, it must be reversible by existing analgesics and validated by multiple independent investigators. In the present study, we demonstrate that inflammation and nerve injury minimally interfere with wheel running, locomotion, gait, social interaction, and anxiety-like behaviors in mice. Although significant nerve injury-induced gait deficits were observed, they were not reversed by the analgesic PD123319. Thus, we conclude that these voluntary behaviors are not reliable pain-related readouts across rodent injury models.

CFA- and SNI-induced changes in physical activity are transient, if present

We tested whether voluntary measures of physical activity and mobility – wheel running, open field locomotion, and gait – are impaired in mice following CFA or SNI. We found that bilateral CFA reduced each measure of physical activity and mobility for up to 3 days post injection, suggesting that CFA transiently suppresses global physical activity in mice. These findings are in close alignment with similar studies of CFA-induced decreases in voluntary wheel running.^{12,24,31,60} In contrast, unilateral SNI impaired gait (see below), but otherwise did not interfere with physical activity or mobility. Previous studies support our finding that open field locomotion is unaffected by nerve injury.^{9,46,87,92,94} However, our data demonstrating that nerve injury does not suppress wheel running raises questions.

A distinguishing feature of our wheel running paradigm is that mice were provided acute (2 hr), rather than homecage (24 hr) wheel access, which could have influenced our null observation. However, existing studies in which rodents were provided homecage wheel access provide conflicting evidence regarding voluntary wheel running as a pain-related behavioral endpoint following nerve injury. Pitzer et al. report that SNI attenuated homecage wheel running distances in mice, and differences between sham and SNI mice were only apparent during the dark cycle.⁶¹ However, Grace et al. observed no difference in homecage running distances between sham and CCI rats²³, and our previous work similarly found no difference between running distances of naïve and SNI mice that were given acute access for 2 or 12 hr/night.⁷³

Another noteworthy feature of our nerve injury studies is that SNI was unilateral. We and others have found that bilateral CFA, but not unilateral CFA, suppresses voluntary wheel running¹². These results suggest that rodents may be better able to adapt to unilateral hindpaw injury compared to bilateral injury. Whether the nerve injury model, injury laterality, testing time of day, or extent of wheel access prior to and/or following induction of pain underlie these discrepancies requires further investigation. Collectively, our present and previous data support that voluntary wheel running, and more broadly physical activity, is reliably suppressed for short a time post-injury by inflammation, but not by nerve injury.

Whether gait alterations represent pain-related/avoidance behaviors across rodent injury models is unclear.^{8,14,46,57,59–61,69,87} We found that both CFA and SNI reduced hindpaw pressure of the affected limb(s). In addition, SNI altered dynamic gait parameters including stance and swing phase, while CFA did not. The use of bilateral CFA versus unilateral SNI may underlie the apparent differences between the effects of inflammation and nerve injury on dynamic gait parameters within our study. In fact, previous rodent gait analysis studies of both unilateral inflammatory and nerve injury pain models show significant changes in stance and swing phases.^{8,14,46,59–61}

Interestingly, we found that compared to the duration of mechanical hypersensitivity, gait alterations following CFA were transient, whereas gait changes following SNI persisted equally as long as mechanical hypersensitivity. As the SNI model involves ligating both sensory and motor axons, we hypothesized that SNI-induced gait changes were due in part to a motor deficit, and our data suggest that this is the case. First, like others⁸⁷, we found that SNI impaired gross motor coordination measured via the rotarod test. Similarly, our previous results show that SNI mice were impaired in the inverted screen test and had significantly atrophied gastrocnemius muscles⁷³, further demonstrating the impact of SNI on motor axons. Second, although nerve injury-induced gait changes have been proposed to reflect pain-related behavior⁶¹, to our knowledge, only one previous study has evaluated the effect of analgesics on Catwalk gait deficits in mice.⁴⁶ We show that SNI-induced gait changes were not reversed by the analgesic PD123319. Together, these findings indicate that nerve injury induced-changes in gait are not driven by pain; rather, they are likely the product of a motor deficit. In contrast, it is likely that pain underlies inflammation-induced changes in gait, which have been successfully reversed by analgesics.^{1,59,69} These observations emphasize that demonstrating reversal via known analgesics is a crucial step in establishing new pain-related behaviors.

Clinically, it is not uncommon for analgesics to reverse hypersensitivity, yet fail to improve other aspects of chronic pain.¹⁹ Indeed, assays used in the present study likely generate differing nociceptive inputs: the von Frey test of mechanical hypersensitivity entails focal hindpaw mechanical stimulation, whereas voluntary behavior assays such as gait analysis or wheel running reflect nociceptive inputs integrated from the entire hindpaw. Thus, it is possible our observation that PD123319 reverses SNI-mechanical hypersensitivity, yet fails to reverse SNI-induced gait deficits, reflects differences in analgesic efficacy of PD123319 on differing nociceptive stimulus inputs. However, this possibility is contradicted by the finding that PD123319 successfully reverses SNI-induced changes in voluntary behavior in assays that

similarly vary in nociceptive inputs, including the warm/cool plate avoidance system as well as the mechanical avoidance assay.⁷⁴

Neither CFA nor SNI alter social interactions

There is growing evidence from clinical and preclinical studies that chronic pain both influences and is influenced by social interactions.^{34,38,39,44,60,61,78} For instance, patients report that pain substantially interferes with social activities and relationships.^{30,53,90} Like humans, mice partake in complex social interactions.^{75,86} The social approach assay utilized here encompasses a combination of perhaps conflicting motivations including social investigation, play, offensive aggression, and/or perception of the stimulus mouse as a stressor.⁵ We found no effect of CFA or SNI on C57BL/6J social interactions on PID 2 or POD 8/14, respectively. Previous studies have similarly shown that social interactions are unchanged in mice following inflammation^{36,87}, and reduced acutely (POD 5) after nerve injury, if at all.^{87,94} These data suggest that changes in social interaction do not reliably manifest in common mouse models of persistent pain. Notably, C57BL/6J mice display high sociability compared to other mouse strains^{5,71}, which may mask the effects of injury on social behavior. Thus, the impact of inflammation and nerve injury on social interaction across strains requires further investigation.

Neither CFA nor SNI induce anxiety-like behavior

There is an increased prevalence of anxiety in chronic pain patients.^{25,30} To test whether persistent pain similarly induces anxiety-like behavior in mice, we utilized the open field test and found that neither CFA nor SNI elicited anxiety-like behavior during ongoing mechanical hypersensitivity. Previous studies using the open field test have also reported a lack of anxiety-like behavior in mice up to 4 weeks after inflammation or nerve injury.^{36,87} However, anxiety-like behavior has been detected in rodents using the elevated plus maze after both inflammation

and nerve injury, and is reversed by anxiolytics and analgesics.^{17,36,50,56,62,70} Therefore, it is possible that CFA and SNI mice would have exhibited anxiety-like behavior if we had used additional measures of anxiety-like behavior.

Hypersensitivity and suppressed voluntary behaviors represent different aspects of pain

We demonstrated that changes in mechanical withdrawal thresholds and voluntary behaviors represent distinct components of inflammation and nerve injury. We found no correlation between CFA- and/or SNI-induced mechanical hypersensitivity and decreased Catwalk hindpaw pressure or voluntary wheel running. Similarly, studies of other rodent voluntary behaviors such as burrowing and sleep cycle report no correlation with hypersensitivity.^{35,47} These results support the idea that changes in paw-withdrawal reflexes and voluntary behavior are driven by different pathophysiologies.^{45,87} For instance, compared to mechanical withdrawal reflexes, voluntary wheel running is a complex behavior that engages reward circuitry.^{4,54} In turn, disruption of this behavior by pain likely reflects a combination of somatosensory, affective, and motivational changes.

Changes in voluntary behavior are not characteristic of persistent pain in mice

Improving the translatability of basic research findings is a priority to the field. In turn, considerable efforts have been directed towards identifying measures of spontaneous pain in rodent models. While impairment of voluntary behaviors and quality of life measures have successfully been reversed by analgesics in rodent models of acute pain^{42,81,82}, the present study and others demonstrate that these endpoints are less informative in common rodent models of persistent pain.^{46,87} In most cases, there are either no or only modest, short-lived changes in voluntary behavior, which limit the ability to study the time course of pain pathologies and the efficacy of novel analgesics. These observations raise two important possibilities.

Foremost, spontaneous pain may be either short-lived or well-masked in mice because as prey animals, it is evolutionarily disadvantageous for mice to display signs of injury or weakness. Second, while mice certainly display sensitization after injury, it is possible that mice do not experience pain as a complex sensory and emotional state as humans do. Both of these possibilities could represent a formidable challenge of using mice to model the negative impact of chronic pain on the quality of life of humans.

Despite these obstacles, there are a variety of promising tools to bridge the translational gap between rodent and human pain research. For instance, operant and classical conditioning assays such as the mechanical conflict system and conditioned place preference/aversion possess predictive validity as measures of motivational aspects of pain.^{26,51} Further, agnostic approaches to analyzing rodent body language and behavioral phenotypes may reveal novel endpoints that access the presence of ongoing sensitization in rodents without anthropomorphizing.^{40,91} Thus, although we found minimal effects of inflammation and nerve injury on mouse physical activity, social interaction, or anxiety-like behavior, using operant assays or agonistic approaches in conjunction with traditional measures of nociceptive thresholds may aid in increasing the translation of preclinical findings.

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2.7 Author Contributions

Designed the experiments: TDS, ERS, MRB, AJS, DPM, RWG, and JPG. Performed the experiments: TDS and JPG. Analyzed the data: TDS and JPG. Wrote the manuscript: TDS. All authors reviewed, edited, and approved the manuscript.

2.8 References

1. Adams BL, Guo W, Gors RT, Knopp KL: Pharmacological interrogation of a rodent forced ambulation model: leveraging gait impairment as a measure of pain behavior pre-clinically. *Osteoarthritis Cartilage* 24:1928–39, 2016.
2. Barrot M: Tests and models of nociception and pain in rodents. *Neuroscience* 211:39–50, 2012.
3. Batka RJ, Brown TJ, Mcmillan KP, Meadows RM, Jones KJ, Haulcomb MM: The need for speed in rodent locomotion analyses. *Anat Rec* 297:1839–64, 2014.
4. Brene S, Bjornebekk A, Aberg E, Mathe AA, Olson L, Werme M: Running is rewarding and antidepressive. *Physiol Behav* 92:136–40, 2007.
5. Brodtkin ES, Hagemann A, Nemetski SM, Silver LM: Social approach-avoidance behavior of inbred mouse strains towards DBA/2 mice. *Brain Res* 1002:151–7, 2004.
6. Burkholder T, Foltz C, Karlsson E, Linton CG, Joanne M: Health Evaluation of Experimental Laboratory Mice. *Curr Protoc Mouse Biol* 2:145–65, 2012.
7. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53:55–63, 1994.
8. Chiang C-Y, Sheu M-L, Cheng F-C, Chen C-J, Su H-L, Sheehan J, Pan C: Comprehensive analysis of neurobehavior associated with histomorphological alterations in a chronic constrictive nerve injury model through use of the CatWalk XT system Laboratory investigation. *J Neurosurg* 120:250–62, 2014.
9. Cho H, Jang Y, Lee B, Chun H, Jung J, Kim SM, Hwang SW, Oh U: Voluntary movements as a possible non-reflexive pain assay. *Mol Pain* 9:25, 2013.
10. Clark JD: Preclinical Pain Research: Can We Do Better? *Anesthesiology* 125:846–9, 2016.
11. Cobiánchi S, Arbat-Plana A, López-Álvarez VM, Navarro X: Neuroprotective Effects of Exercise Treatments After Injury: The Dual Role of Neurotrophic Factors. *Curr Neuropharmacol* 15:495–518, 2017.
12. Cobos EJ, Ghasemlou N, Araldi D, Segal D, Duong K, Woolf CJ: Inflammation-induced decrease in voluntary wheel running in mice: A nonreflexive test for evaluating inflammatory pain and analgesia. *Pain* 153:876–84, 2012.
13. Cobos EJ, Portillo-Salido E: “Bedside-to-Bench” Behavioral Outcomes in Animal Models of Pain: Beyond the Evaluation of Reflexes. *Curr Neuropharmacol* 11:560–91, 2013.
14. Coulthard P, Pleu BJ, Brewster M, Wilson L, Macfarlane T V: Gait analysis as an objective measure in a chronic pain model. *J Neurosci Methods* 116:197–213, 2002.
15. Cummins RA, Walsh RN: The Open-Field Test: A Critical Review. *Physiol Bull* 83:482–504, 1976.
16. Decosterd I, Woolf CJ: Spared nerve injury: an animal model of persistent peripheral

neuropathic pain. *Pain* 87:149–58, 2000.

17. Dimitrov EL, Tsuda MC, Cameron HA, Usdin TB: Anxiety- and depression-like behavior and impaired neurogenesis evoked by peripheral neuropathy persist following resolution of prolonged tactile hypersensitivity. *J Neurosci* 34:12304–12, 2014.
18. Duraku LS, Hossaini M, Hoendervangers S, Falke LL, Kambiz S, Mudera VC, Holstege JC, Walbeehm ET, Ruigrok TJH: Spatiotemporal dynamics of re-innervation and hyperinnervation patterns by uninjured CGRP fibers in the rat foot sole epidermis after nerve injury. *Mol Pain* 8:61, 2012.
19. Dworkin RH, Turk DC, Farrar JT, Haythornthwaite JA, Jensen MP, Katz NP, Kerns RD, Stucki G, Allen RR, Bellamy N, Carr DB, Chandler J, Cowan P, Dionne R, Galer BS, Hertz S, Jadad AR, Kramer LD, Manning DC, Martin S, McCormick CG, McDermott MP, McGrath P, Quessy S, Rappaport BA, Robbins W, Robinson JP, Rothman M, Royal MA, Simon L, Stauffer JW, Stein W, Tollett J, Wernicke J, Witter J: Core outcome measures for chronic pain clinical trials: IMMPACT recommendations. *Pain* 113:9–19, 2005.
20. Dysvik E, Lindstrøm TC, Eikeland OJ, Natvig GK: Health-related quality of life and pain beliefs among people suffering from chronic pain. *Pain Manag Nurs* 5:66–74, 2004.
21. Gabriel AF, Marcus MAE, Honig WMM, Walenkamp GHIM, Joosten EAJ: The CatWalk method: a detailed analysis of behavioral changes after acute inflammatory pain in the rat. *J Neurosci Methods* 163:9–16, 2007.
22. Gereau RW, Sluka KA, Maixner W, Savage SR, Price TJ, Murinson BB, Sullivan MD, Fillingim RB: A pain research agenda for the 21st century. *J Pain* 15:1203–14, 2014.
23. Grace PM, Fabisiak TJ, Green-Fulham SM, Anderson ND: Prior voluntary wheel running attenuates neuropathic pain. *Pain* 157:2012–23, 2016.
24. Grace PM, Strand KA, Maier SF, Watkins LR: Suppression of voluntary wheel running in rats is dependent on the site of inflammation: evidence for voluntary running as a measure of hind paw-evoked pain. *J Pain* 15:121–8, 2014.
25. Gureje O: Comorbidity of pain and anxiety disorders. *Curr Psychiatry Rep* 10:318–22, 2008.
26. Harte SE, Meyers JB, Donahue RR, Taylor BK, Morrow TJ: Mechanical Conflict System: A Novel Operant Method for the Assessment of Nociceptive Behavior. *PLoS One* 11:e0150164, 2016.
27. He D, Grant B, Holden RR, Gilron I: Methodology for self-report of rest pain (or spontaneous pain) vs evoked pain in chronic neuropathic conditions. *PAIN Reports* 2:e587, 2017.
28. Iadarola MJ, Douglass J, Civelli O, Naranjo JR: Differential activation of spinal cord dynorphin and enkephalin neurons during hyperalgesia: evidence using cDNA hybridization. *Res Reports* 455:205–12, 1988.
29. Institute of Medicine: *Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research*. 2011.

30. Jensen M, Chodroff M, Dworkin R: The impact of neuropathic pain on health-related quality of life: review and implications. *Neurology* 68:1178–82, 2007.
31. Kandasamy R, Calsbeek JJ, Morgan MM: Home cage wheel running is an objective and clinically relevant method to assess inflammatory pain in male and female rats. *J Neurosci Methods* 263:115–22, 2016.
32. Kissin I: The development of new analgesics over the past 50 years: A lack of real breakthrough drugs. *Anesth Analg* 110:780–9, 2010.
33. Koltyn KF: Analgesia Following Exercise: A Review. *Sports Med* 29:85–98, 2000.
34. Langford DJ, Tuttle AH, Brown K, Deschenes S, Fischer DB, Mutso A, Root KC, Sotocinal SG, Stern MA, Mogil JS, Sternberg WF: Social approach to pain in laboratory mice. *Soc Neurosci* 5:163–70, 2010.
35. Lau W, Dykstra C, Thevarkunnel S, Silenieks LB, de Lannoy IAM, Lee DKH, Higgins GA: A back translation of pregabalin and carbamazepine against evoked and non-evoked endpoints in the rat spared nerve injury model of neuropathic pain. *Neuropharmacology* 73:204–15, 2013.
36. Liu Y, Yang L, Yu J, Zhang Y-Q: Persistent, comorbid pain and anxiety can be uncoupled in a mouse model. *Physiol Behav* 151:55–63, 2015.
37. Mao J: Current challenges in translational pain research. *Trends Pharmacol Sci* 33:568–73, 2012.
38. Martin LJ, Hathaway G, Isbester K, Mirali S, Acland EL, Niederstrasser N, Slepian PM, Trost Z, Bartz JA, Sapolsky RM, Sternberg WF, Levitin DJ, Mogil JS: Reducing Social Stress Elicits Emotional Contagion of Pain in Mouse and Human Strangers. *Curr Biol* 25:326–32, 2015.
39. Martin LJ, Tuttle AH, Mogil JS: The Interaction Between Pain and Social Behavior in Humans and Rodents. *Curr Top Behav Neurosci* 20:233–50, 2014.
40. McCall JG, Hammarsten C, Bord A, Sheahan TD, Golden JP, Gereau IV RW: Agnostic identification of mouse behavioral phenotypes. *J Pain* 18:S13, 2017.
41. McNamee P, Mendolia S: The effect of chronic pain on life satisfaction: Evidence from Australian data. *Soc Sci Med* 121:65–73, 2014.
42. Miller LL, Picker MJ, Schmidt KT, Dykstra LA: Effects of morphine on pain-elicited and pain-suppressed behavior in CB1 knockout and wildtype mice. *Psychopharmacology (Berl)* 215:455–65, 2011.
43. Mogil JS: Animal models of pain: progress and challenges. *Nat Rev Neurosci* 10:283–94, 2009.
44. Mogil JS: Social modulation of and by pain in humans and rodents. *Pain* 156:S35–41, 2015.
45. Mogil JS, Crager SE: What should we be measuring in behavioral studies of chronic pain in animals? *Pain* 112:12–5, 2004.

46. Mogil JS, Graham AC, Ritchie J, Hughes SF, Austin J-S, Schorscher-Petcu A, Langford DJ, Bennett GJ: Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. *Mol Pain* 6:34, 2010.
47. Monassi CR, Bandler R, Keay KA: A subpopulation of rats show social and sleep-waking changes typical of chronic neuropathic pain following peripheral nerve injury. *Eur J Neurosci* 17:1907–20, 2003.
48. Moore A, Derry S, Eccleston C, Kalso E: Expect analgesic failure; pursue analgesic success. *Br Med J* 346:f2690, 2013.
49. Muralidharan A, Wyse BD, Smith MT: Analgesic efficacy and mode of action of a selective small molecule angiotensin II type 2 receptor antagonist in a rat model of prostate cancer-induced bone pain. *Pain Med* 15:93–110, 2014.
50. Narita M, Kaneko C, Miyoshi K, Nagumo Y, Kuzumaki N, Nakajima M, Nanjo K, Matsuzawa K, Yamazaki M, Suzuki T: Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. *Neuropsychopharmacology* 31:739–50, 2006.
51. Navratilova E, Xie JY, King T, Porreca F: Evaluation of reward from pain relief. *Ann N Y Acad Sci* :1–11, 2013.
52. Negus SS, Vanderah TW, Brandt MR, Bilsky EJ, Becerra L, Borsook D: Preclinical Assessment of Candidate Analgesic Drugs : Recent Advances and Future Challenges. *J Pharmacol Exp Ther* 319:507–14, 2006.
53. Nicholson B, Verma S: Comorbidities in chronic neuropathic pain. *Pain Med* 5:S9–27, 2004.
54. Novak CM, Burghardt PR, Levine JA: The use of a running wheel to measure activity in rodents: Relationship to energy balance, general activity, and reward. *Neurosci Biobehav Rev* 36:1001–14, 2012.
55. O'Connor AB: Neuropathic Pain: Quality-of-Life Impact, Costs, and Cost Effectiveness of Therapy. *Pharmacoeconomics* 27:95–112, 2009.
56. Parent AJ, Beaudet N, Beaudry H, Bergeron J, Bérubé P, Drolet G, Sarret P, Gendron L: Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain. *Behav Brain Res* 229:160–7, 2012.
57. Parvathy SS, Masocha W: Gait analysis of C57BL/6 mice with complete Freund's adjuvant-induced arthritis using the CatWalk system. *BMC Musculoskelet Disord* 14:14, 2013.
58. Pechlivanova DM, Markova PP, Popov D, Stoynev AG: Peptides The role of the angiotensin AT2 receptor on the diurnal variations of nociception and motor coordination in rats. *Peptides* 39:152–6, 2013.
59. Piesla MJ, Leventhal L, Strassle BW, Harrison JE, Cummons TA, Lu P, Whiteside GT: Abnormal gait, due to inflammation but not nerve injury, reflects enhanced nociception in preclinical pain models. *Brain Res* 1295:89–98, 2009.

60. Pitzer C, Kuner R, Tappe-Theodor A: Voluntary and evoked behavioral correlates in inflammatory pain conditions under different social housing conditions. *Pain Reports* 1:e564, 2016.
61. Pitzer C, Kuner R, Tappe-Theodor A: Voluntary and evoked behavioral correlates in neuropathic pain states under different social housing conditions. *Mol Pain* 12:1–15, 2016.
62. Refsgaard LK, Hoffmann-Petersen J, Sahlholt M, Pickering DS, Andreassen JT: Modelling affective pain in mice: Effects of inflammatory hypersensitivity on place escape/avoidance behaviour, anxiety and hedonic state. *J Neurosci Methods* 262:85–92, 2016.
63. Ren K, Dubner R: Inflammatory Models of Pain and Hyperalgesia. *ILAR J* 40:111–8, 1999.
64. Ren W, Centeno MV, Berger S, Wu Y, Na X, Liu X, Kondapalli J, Apkarian AV, Martina M, Surmeier DJ: The indirect pathway of the nucleus accumbens shell amplifies neuropathic pain. *Nat Neurosci* 19:220–2, 2015.
65. Rice ASC, Cimino-Brown D, Eisenach JC, Kontinen VK, Lacroix-Fralish ML, Machin I, Mogil JS, Stoehr T: Comment on: Animal models and the prediction of efficacy in clinical trials of analgesic drugs: A critical appraisal and a call for uniform reporting standards. *Pain* 139:243–7, 2009.
66. Rice ASC, Dworkin RH, McCarthy TD, Anand P, Bountra C, McCloud PI, Hill J, Cutter G, Kitson G, Desem N, Raff M: EMA401, an orally administered highly selective angiotensin II type 2 receptor antagonist, as a novel treatment for postherpetic neuralgia: A randomised, double-blind, placebo-controlled phase 2 clinical trial. *Lancet* 383:1637–47, 2014.
67. Richner M, Bjerrum OJ, Nykjaer A, Vaegter CB: The spared nerve injury (SNI) model of induced mechanical allodynia in mice. *J Vis Exp* :e3092, 2011.
68. Rigaud M, Gemes G, Barabas M-E, Chernoff DI, Abram SE, Stucky CL, Hogan QH: Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. *Pain* 136:188–201, 2008.
69. Robinson I, Sargent B, Hatcher JP: Use of dynamic weight bearing as a novel end-point for the assessment of Freund's Complete Adjuvant induced hypersensitivity in mice. *Neurosci Lett* 524:107–10, 2012.
70. Roeska K, Doods H, Arndt K, Treede R-D, Ceci A: Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin. *Pain* 139:349–57, 2009.
71. Sankoorikal GM V, Kaercher KA, Boon CJ, Lee JK, Brodtkin ES: A mouse model system for genetic analysis of sociability: C57BL/6J versus BALB/cJ inbred mouse strains. *Biol Psychiatry* 59:415–23, 2006.
72. Sapunar D, Puljak L: What can rats tell us about neuropathic pain? Critical evaluation of behavioral tests used in rodent pain models. *Period Biol* 111:155–60, 2009.
73. Sheahan TD, Copits BA, Golden JP, Gereau RW: Voluntary Exercise Training: Analysis

- of Mice in Uninjured, Inflammatory, and Nerve-Injured Pain States. *PLoS One* 10:e0133191, 2015.
74. Shepherd AJ, Mickle AD, Copits BA, Karlsson P, Kadunganattil S, Golden JP, Tadinada SM, Mack MR, Haroutounian S, de Kloet AD, Samineni VK, Valtcheva M V, McIlvried LA, Sheahan TD, Jain S, Ray PR, Usachev YM, Dussor G, Kim BS, Krause EG, Price TJ, Gereau RW, Mohapatra DP: Macrophage-to-sensory neuron crosstalk mediated by Angiotensin II type-2 receptor elicits neuropathic pain. *bioRxiv* , 2017.
 75. Silverman JL, Yang M, Lord C, Crawley JN: Behavioural phenotyping assays for mouse models of autism. *Nat Rev Neurosci* 11:490–502, 2010.
 76. Siuda ER, Al-Hasani R, McCall JG, Bhatti DL, Bruchas MR: Chemo- and Optogenetic Activation of G α s Signaling in the Basolateral Amygdala Induces Acute and Social Anxiety-Like States. *Neuropsychopharmacology* 41:2011–3, 2016.
 77. Smith BH, Torrance N: Epidemiology of neuropathic pain and its impact on quality of life. *Curr Pain Headache Rep* 16:191–8, 2012.
 78. Smith ML, Hostetler CM, Heinricher MM, Ryabinin AE: Social transfer of pain in mice. *Sci Adv* 2:e160085, 2016.
 79. Smith MT, Lau T, Wallace VCJ, Wyse BD, Rice ASC: Analgesic efficacy of small-molecule angiotensin II type 2 receptor antagonists in a rat model of antiretroviral toxic polyneuropathy. *Behav Pharmacol* 25:137–46, 2014.
 80. Smith MT, Muralidharan A: Targeting angiotensin II type 2 receptor pathways to treat neuropathic pain and inflammatory pain. *Expert Opin Ther Targets* 19:25–35, 2015.
 81. Stevenson GW, Bilsky EJ, Negus SS: Targeting Pain-Suppressed Behaviors in Preclinical Assays of Pain and Analgesia: Effects of Morphine on Acetic Acid-Suppressed Feeding in C57BL/6J Mice. *J Pain* 7:408–16, 2006.
 82. Stevenson GW, Cormier J, Mercer H, Adams C, Dunbar C, Negus SS, Bilsky EJ: Targeting pain-depressed behaviors in preclinical assays of pain and analgesia : Drug effects on acetic acid-depressed locomotor activity in ICR mice. *Life Sci* 85:309–15, 2009.
 83. Stevenson GW, Mercer H, Cormier J, Dunbar C, Benoit L, Adams C, Jezierski J, Luginbuhl A, Bilsky EJ: Monosodium iodoacetate-induced osteoarthritis produces pain-depressed wheel running in rats: Implications for preclinical behavioral assessment of chronic pain. *Pharmacol Biochem Behav* 98:35–42, 2011.
 84. Sullivan MD, Ballantyne JC: Must we reduce pain intensity to treat chronic pain? *Pain* 157:65–9, 2016.
 85. Tappe-Theodor A, Kuner R: Studying ongoing and spontaneous pain in rodents - challenges and opportunities. *Eur J Neurosci* 39:1881–90, 2014.
 86. Terranova ML, Laviola G: Scoring of social interactions and play in mice during adolescence. *Curr Protoc Toxicol* Chapter 13:13.10.1-13.10.11, 2005.
 87. Urban R, Scherrer G, Goulding EH, Tecott LH, Basbaum AI: Behavioral indices of

- ongoing pain are largely unchanged with tissue or nerve-injury induced mechanical hypersensitivity. *Pain* 152:990–1000, 2011.
88. Vierck CJ, Hansson PT, Yeziarski RP: Clinical and pre-clinical pain assessment: are we measuring the same thing? *Pain* 135:7–10, 2008.
 89. Vrinten DH, Hamers FFT: “CatWalk” automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing. *Pain* 102:203–9, 2003.
 90. Wallace LS, Wexler RK, McDougale L, Miser WF, Haddox JD: Voices that may not otherwise be heard: A qualitative exploration into the perspectives of primary care patients living with chronic pain. *J Pain Res* 7:291–9, 2014.
 91. Wiltchko AB, Johnson MJ, Iurilli G, Peterson RE, Katon JM, Pashkovski SL, Abaira VE, Adams RP, Datta SR: Mapping Sub-Second Structure in Mouse Behavior. *Neuron* 88:1121–35, 2015.
 92. Yalcin I, Bohren Y, Waltisperger E, Sage-Ciocca D, Yin JC, Freund-Mercier M-J, Barrot M: A Time-Dependent History of Mood Disorders in a Murine Model of Neuropathic Pain. *Biol Psychiatry* 70:946–53, 2011.
 93. Ye G-L, Savelieva K V, Vogel P, Baker KB, Mason S, Lanthorn TH, Rajan I: Ligation of mouse L4 and L5 spinal nerves produces robust allodynia without major motor function deficit. *Behav Brain Res* 276:99–110, 2014.
 94. Zhou W, Dantzer R, Budac DP, Walker AK, Mao-Ying Q-L, Lee AW, Heijnen CJ, Kavelaars A: Peripheral indoleamine 2,3-dioxygenase 1 is required for comorbid depression-like behavior but does not contribute to neuropathic pain in mice. *Brain Behav Immun* 46:147–53, 2015.

Chapter 3

Metabotropic glutamate receptor 2/3 (mGluR2/3) activation suppresses
TRPV1 sensitization in mouse, but not human sensory neurons

3.1 Abstract

The use of human tissue to validate putative analgesic targets identified in rodents is a promising strategy for improving the historically poor translational record of preclinical pain research. We recently demonstrated that in mouse and human sensory neurons, agonists for metabotropic glutamate receptors 2 and 3 (mGluR2/3) reduce membrane hyperexcitability produced by the inflammatory mediator prostaglandin E₂ (PGE₂). Previous rodent studies indicate that mGluR2/3 can also reduce peripheral sensitization by suppressing inflammation-induced sensitization of TRPV1, but to date, whether this observation similarly translates to human sensory neurons has not been tested. Here, we demonstrate using calcium imaging that activation of mGluR2/3 with the agonist APDC suppresses PGE₂-induced sensitization of TRPV1 in mouse, but not human sensory neurons. We also evaluate sensory neuron expression of the gene transcripts for mGluR2 (*Grm2*), mGluR3 (*Grm3*), and TRPV1 (*Trpv1*) using *in situ* hybridization. We show that the majority of *Trpv1*⁺ mouse and human sensory neurons express *Grm2* and/or *Grm3*, and in both mouse and human, *Grm2* was expressed in a greater percentage of sensory neurons than *Grm3*. Although our calcium imaging studies demonstrated a functional difference in the modulation of TRPV1 sensitization by mGlu2/3 activation between mouse and human, there were no species differences in the gene transcript colocalization of mGluR2 or mGluR3 with TRPV1 that might explain this functional difference. Taken together with our previous work, these results suggest that activation of mGluR2/3 suppresses some, but not all, aspects of human sensory neuron sensitization caused by PGE₂. These differences have implications for potential future healthy human voluntary studies or clinical trials that seek to assess the analgesic efficacy of mGluR2/3 agonists or positive allosteric modulators.

3.2 Introduction

Species differences between rodents and humans have been proposed to contribute to the low success rate of analgesic drug development.^{6,43,67,68} In preclinical research, putative analgesics are oftentimes identified and exclusively evaluated in rodent tissues and/or pain models prior to entering clinical trials, in which such drugs seldom demonstrate efficacy.^{38,47,68} Therefore, using primary human neurons to validate preclinical rodent findings is an appealing strategy to improve the translational success of basic pain research findings. With this goal in mind, we and others have established approaches to obtain and utilize human sensory neurons to better understand human nociceptor physiology.^{1,8,22,32,64,74}

The group II metabotropic glutamate receptors (mGluRs) have recently been identified as putative targets for pain relief in rodents.^{3,14,15,19,26,36,37,39,51,58,59,70–72} mGluR2 and mGluR3 are seven transmembrane domain G_i-protein coupled receptors that decrease cAMP formation, activate potassium channels, and inhibit voltage-gated calcium channels to reduce neuronal excitability and synaptic transmission^{21,35}. While group II mGluRs are expressed at each level of the pain neuraxis^{11–13,19,23,39,52,60,65}, several lines of evidence suggest that activation of mGlu2 and mGlu3 receptors in peripheral sensory neurons is sufficient for analgesia. For instance, in rodent inflammatory and neuropathic pain models, pharmacological activation of mGluR2/3 expressed on peripheral primary afferents can attenuate pain-like behavior by suppressing sensory neuron sensitization in response to algogens and inflammatory mediators.^{3,14,15,23,26,70} Conversely, pharmacological inhibition of peripheral mGluR2/3 can prolong pain-like behavior and increase sensory neuron activity, suggesting endogenous activation of mGluR2/3 is analgesic.^{15,71} Given the centrally mediated adverse effects of existing analgesics such as opioid addiction and abuse, peripheral analgesic targets are of particular interest.

Our recent studies on cultured human dorsal root ganglia (DRG) neurons suggest peripheral mGlu2/3 receptors may be clinically relevant analgesic targets. We demonstrated

both anatomical and functional expression of group II mGluRs in human DRG.²³ Importantly, as in mouse, mGluR2/3 activation blocked human nociceptor membrane hyperexcitability produced by the inflammatory mediator prostaglandin E₂ (PGE₂), indicating that a mechanism for peripheral analgesia may be conserved across species.²³ Rodent studies suggest mGlu2/3 receptors expressed on sensory neuron peripheral terminals can also reduce sensory neuron sensitization by suppressing sensitization of TRPV1^{14,15,26,70}, a nonselective cation channel that detects noxious stimuli and is critical for inflammation-induced peripheral sensitization.^{16,17,24,48} The present study tested whether the same mechanism is conserved in human. We utilized sensory neurons obtained from organ donors without chronic pain to determine whether mGluR2/3 activation blocks inflammation-induced sensitization of TRPV1 in human neurons. We demonstrate that group II mGluR activation suppresses PGE₂-induced sensitization of TRPV1 calcium responses in mouse, but not human sensory neurons. Interestingly, these functional differences were not explained by species differences in coexpression of the TRPV1 gene transcript with mGlu2 or mGlu3 receptor gene transcripts.

3.3 Materials and Methods

Animals

All experiments were performed in compliance with protocols approved by the Animal Studies Committee of Washington University in St. Louis (Protocol Numbers 20150246 and 20160097). Experiments were conducted on 5-8 week old C57BL/6J male and female mice (Jackson Lab, Bar Harbor, ME). Mice were housed in an animal facility with a 12-hour light-dark cycle and given food and water *ad libitum*.

Donors

Human tissue was obtained in compliance with procedures approved by Mid-America Transplant (St. Louis, MO), and the Human Research Protection Office at Washington University in St. Louis provided an International Review Board waiver. Human dorsal root ganglia (DRG) were obtained from organ donors with full legal consent for use of tissue for research. Only donors without a history of chronic pain were used in this study (Table 1).

Table 1. Donor information and tissue

Donor	Age, y	Sex	Race	Cause of Death	Tissue Uses
1	21	Male	White	Stroke	Ca ²⁺ imaging
2	55	Female	White	Stroke	Ca ²⁺ imaging, FISH
3	22	Male	Black	Anoxia	Ca ²⁺ imaging
4	44	Female	White	Stroke	Ca ²⁺ imaging
5	12	Female	White	Anoxia	Ca ²⁺ imaging, FISH
6	26	Male	White	Head Trauma	Ca ²⁺ imaging, FISH
7	18	Female	White	Head Trauma	Ca ²⁺ imaging, FISH
8	18	Male	White	Head Trauma	Ca ²⁺ imaging, FISH

y, year; FISH, fluorescent *in situ* hybridization

Mouse DRG cultures

Each cell culture reagent vendor and catalog number has been listed previously.⁶⁴ For each tissue preparation, two age- and sex-matched mice were sacrificed by live decapitation,

and cervical through lumbar DRG were removed and pooled together. DRG were incubated in papain (45 U) for 20 min at 37°C, 5% CO₂. DRG were then rinsed and incubated in collagenase (1.5 mg/mL) for 20 min. Both enzyme solutions were made up in Ca²⁺- and Mg²⁺-free Hank's buffered salt solution with 10 mM HEPES. DRG were manually triturated with fire-polished Pasteur pipettes to dissociate neurons, passed through a 40-µm filter, and plated onto poly-D-lysine/collagen coated 12 mm glass coverslips. Neurons were maintained in culture for 2 days in Neurobasal A media supplemented with 100 U/mL penicillin/streptomycin, 2 mM GlutaMAX, B27, and 5% fetal bovine serum.

Human DRG cultures

Human DRG from the first through the fifth lumbar vertebra were extracted and cultured as described in detail previously.⁶⁴ Briefly, following surgical extraction, fat and dura were trimmed away from the ganglia. DRG were minced and incubated in papain for 1 hr, rinsed, and incubated in collagenase for 1 hr. Both enzyme solutions were made up in an *N*-methyl-D-glucamine artificial cerebrospinal fluid solution. DRG were manually triturated with fire-polished Pasteur pipettes to dissociate neurons, passed through a 100-µm filter, and plated onto poly-D-lysine/collagen coated 12 mm glass coverslips. Neurons were maintained in culture for up to 9 days in the media described above. Every 3 days, half of the culture media was replaced with fresh media to ensure neuronal health.

Calcium imaging

Calcium imaging was performed as described previously with modifications.^{63,64} Cultured neurons from mouse and human were incubated with 3 µg/mL of the ratiometric calcium indicator Fura-2 AM (Life Technologies, Carlsbad, CA) for 45 min. Neurons were then incubated in external solution for 15 min to allow for deesterification of Fura-2 AM. External solution

consisted of (in mM): 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 30 Glucose, and 10 HEPES. For recordings, coverslips were placed into a chamber and perfused with room temperature external solution. Cells were viewed under an inverted microscope (Olympus Optical, Tokyo, Japan) and fluorescent images were acquired every 2 s using a Hamamatsu ORCA camera (Hamamatsu, Bridgewater, NJ). SimplePCI Software (HCLImage, Hamamatsu Corporation, Sewickley, PA) was used to identify regions of interest surrounding Fura-2 AM loaded neurons a priori and to record fluorescence emission at alternating excitation wavelengths of 357 and 380 nm.

The experimental protocol entailed a 2 min baseline in external solution followed by a 20 s bath application of 100 nM capsaicin (Sigma, St. Louis, MO), a 3 min wash of external solution, then a treatment condition entailing application of either 1) 7 min of vehicle (external solution), 6 min of 1 μ M prostaglandin E₂ (PGE₂, Tocris, Minneapolis, MN), or 1 min of 10 μ M (2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC, Tocris) alone followed by 6 min of 10 μ M APDC plus 1 μ M PGE₂. Immediately after treatment, a second pulse of 100 nM capsaicin was bath applied, neurons were washed for 6 minutes with external solution, and a 10 s pulse of 50 mM KCl was applied to test for cell viability. At least 2 treatment conditions were tested for a given mouse and donor tissue preparation. All drugs were diluted in external solution. Stock solutions of 2.8 mM PGE₂ and 100 mM APDC were made up in DMSO and water, respectively. Peak calcium responses were calculated by dividing the absolute increase in Fura-2 AM signal following stimulus application by the preceding 30 s baseline Fura-2 AM signal. The response threshold to capsaicin was defined as an increase \geq 10% from baseline signal. Cells that did not respond to high KCl were excluded from calcium imaging analysis.

Fluorescent in situ hybridization (RNAscope)

At the conclusion of mouse and human calcium imaging experiments, neurons were fixed on ice with 4% paraformaldehyde/4% sucrose for 15 min and washed with phosphate

buffered saline (PBS). Fluorescent *in situ* hybridization studies were performed according to the protocol for cultured adherent cells using the RNAscope Multiplex Fluorescent Assay (Advanced Cell Diagnostics, Newark, CA) with minor modifications. Following dehydration and rehydration of cells in ethanol, glass coverslips were mounted onto glass slides using ethyl cyanoacrylate. Neurons were treated with protease III diluted 1:10 (mouse) or 1:5-7.5 (human) at room temperature for 10 min. Species-specific target probes for *Trpv1*, *Grm2*, and *Grm3* (Table 2) were combined, applied to neurons, and allowed to hybridize for 2 hr at 40°C in a humidified oven. A series of incubations were then performed to amplify hybridized probe signal and label target probes with the assigned fluorescence detection channel (C1-C3). Coverslips were counterstained with DAPI using ProLong Gold Antifade Mountant (Invitrogen, Waltham, MA). Neurons were imaged at 40X using a Leica SPE confocal microscope. Fields of interest were identified in the DAPI channel. Fiji (Image J, NIH) software was used to calculate neuron diameter and manually quantify single RNA molecule signals. In the RNAscope assay, each punctate dot represents a single target RNA molecule. However, to reduce the likelihood of false positives, mouse and human neurons were defined as positive for a given RNA target if they had ≥ 4 puncta or ≥ 2 puncta, respectively, based on the range in neuron puncta density observed for each species.

Figure 2. RNAscope probes used for fluorescent in situ hybridization

Target	Catalog Number
Mm-Trpv1	313331
Mm-Grm2-C3	317831-C3
Mm-Grm3-C2	317821-C2
Positive Control Probe-Mm	320881
Hs-TRPV1	415381
Hs-GRM2-C3	589771-C3
Hs GRM3-C2	500181-C2
Positive Control Probe-Hs	320861
Negative Control Probe	320871

Mm, mus musculus; Hs, homo sapiens; C2, channel 2; C3, channel 3

Statistical analyses

The experimenter was blind to treatment condition and gene of interest throughout analysis of calcium imaging and *in situ* hybridization data, respectively. Microsoft Excel, GraphPad Prism (La Jolla, CA), R, and the R package lme4 were used for data organization and statistical analyses.^{7,61} Calcium imaging data were analyzed using 1) unpaired t-tests and Bonferroni correction for multiple comparisons and 2) linear mixed-effects model analyses. Linear mixed-effects model analyses controlled for donor as a random-effect and the sex and age of donors as fixed-effects. As the primary objective of the present study was to make species comparisons, we were not sufficiently powered to evaluate the effects of mouse age or sex on calcium imaging outcomes. Species comparisons of the percentage of capsaicin-responsive neurons and gene transcript expression were made using chi-square tests and Bonferroni correction for multiple comparisons, when appropriate. When describing and discussing species differences in gene transcript expression, we default to mouse mRNA nomenclature.

3.4 Results

mGluR2/3 suppress PGE₂-induced TRPV1 sensitization in mouse, but not human sensory neurons

TRPV1 is sensitized by the cAMP/PKA pathway, which is stimulated by inflammatory mediators such as PGE₂.^{10,41,44,46,53} In contrast, group II mGlu receptor activation inhibits adenylyl cyclase and subsequent cAMP production.^{21,35} To determine whether mGluR2/3 activation blocks TRPV1 sensitization, we quantified capsaicin-induced calcium responses of mouse and human sensory neurons. Two 20 s pulses of 100 nM capsaicin were bath applied to DRG neurons and the degree of TRPV1 sensitization was defined as the response ratio of the peak of the second capsaicin response divided by the peak of the first capsaicin response. Under vehicle conditions, TRPV1 desensitization is observed in both mouse and human DRG neurons in the form of reduced calcium responses to subsequent capsaicin pulses (Fig. 1A, B). In mouse sensory neurons, bath application of PGE₂ between capsaicin pulses significantly increased the capsaicin response ratio compared to vehicle (Fig. 1A, C). Coapplication of the selective group II mGluR agonist APDC with PGE₂ significantly reduced the response ratio compared to PGE₂ alone (Fig. 1A, C). These findings confirm our previously published work in cultured sensory neurons obtained from CD-1 mice in which we also demonstrated that suppression of PGE₂-induced TRPV1 sensitization by APDC is blocked by the group II mGluR antagonist LY341495, and thus is attributable to mGlu2/3 receptor activation.⁷⁰

In human sensory neurons, we found that while application of PGE₂ between capsaicin pulses significantly increased the capsaicin response ratio compared to vehicle, the response ratio following coapplication of APDC with PGE₂ did not significantly differ from application of PGE₂ alone (Fig. 1B, D). Unlike rodents used in preclinical studies, human organ donors display wide demographic and genetic diversity. To determine whether a given organ donor was driving

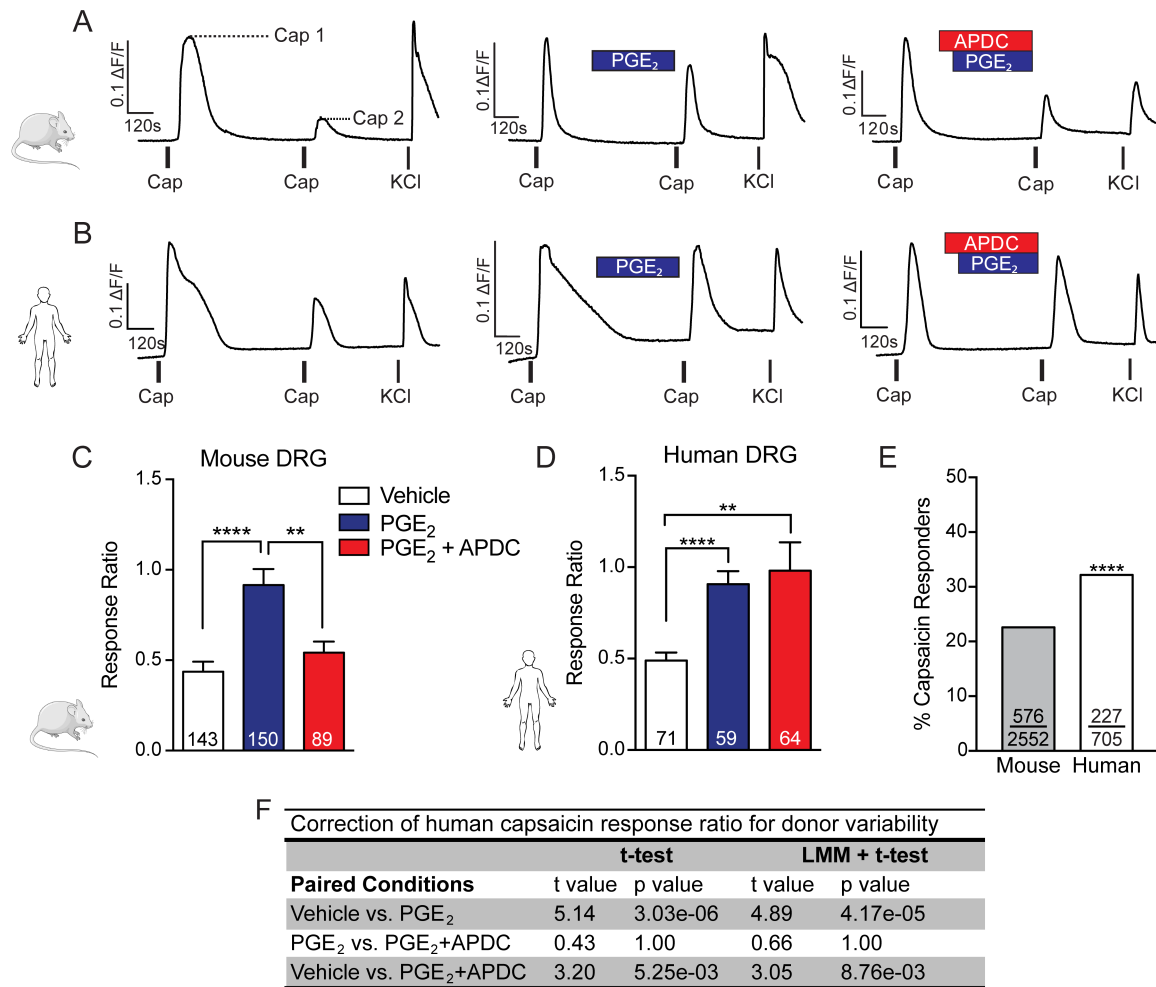


Figure 1. mGlu2/3 receptor activation blocked PGE₂-induced TRPV1 sensitization in mouse, but not human sensory neurons. Representative traces of 100 nM capsaicin-induced calcium responses in mouse (A) and human (B) dorsal root ganglia (DRG) neurons in response to vehicle (left), 1 μ M PGE₂ (middle), or 1 μ M PGE₂ + 10 μ M APDC (right). Experiments concluded with a pulse of 50 mM KCl to determine cell viability. The degree of TRPV1 sensitization is expressed as a response ratio calculated by dividing the peak amplitude of Cap 2 by the peak amplitude of Cap 1 (A, dashed lines). (C) In mouse DRG neurons, PGE₂ significantly increased the capsaicin response ratio compared to vehicle (****p<0.0001; n=143-150 neurons, N=4 preps/condition). Coapplication of APDC with PGE₂ blocked this effect, and significantly reduced the response ratio compared to PGE₂ alone (**p=0.0081; n=89-150 neurons, N=3-4 preps/condition). (D) PGE₂ also significantly increased the capsaicin response ratio of human DRG neurons compared to vehicle (****p<0.0001; n=59-71 neurons, N=5-6 donors/condition); whereas coapplication of APDC did not suppress PGE₂-induced increases in the capsaicin response ratio (p=1, n=59-64 neurons, N=6 donors/condition), which remained significantly greater than vehicle (**p=0.0053, n=64-71 neurons, N=5-6 donors/condition). Capsaicin response ratios were compared using unpaired t-tests and a Bonferroni correction for multiple comparisons. (E) Compared to mouse, a greater percentage of human DRG neurons responded to 100 nM capsaicin (chi square test, $\chi^2=27.27$, ****p<0.0001, mouse: 576/2552 neurons, N=4 preps, human: 227/705 neurons, N=8 donors). (F) Linear mixed-effect model (LMM) regression correction for impact of individual donor, as well as donor age and sex, did not alter human capsaicin response ratio analysis statistical outcomes compared with t-tests alone. Data are presented as mean \pm SEM.

our finding that mGluR2/3 activation fails to suppress PGE₂-induced TRPV1 sensitization in human DRG neurons, we performed a linear mixed-effect model (LMM) regression to correct for donor variability. As the effect of age and sex on human sensory neuron physiology is of broad interest to pain researchers, these parameters were included as covariates in our LMM regression. Interestingly, we found that correcting for impact of individual donor, as well as donor age and sex, did not alter our calcium imaging outcome (Fig. 1F). Taken together, these results demonstrate that mGluR2/3 activation suppresses PGE₂-induced TRPV1 sensitization in mouse, but not human sensory neurons.

We were also interested in whether the same percentage of mouse and human sensory neurons respond to capsaicin. Of the total number of sensory neurons evaluated in calcium imaging studies, 22.6% of mouse sensory neurons versus 32.2% of human sensory neurons responded to 100 nM capsaicin, indicating that human sensory neurons are modestly more capsaicin-responsive (Fig. 1E). These results are consistent with our initial observations that human sensory neurons exhibit greater chemosensitivity to algogens and pruritogens compared to rodents.²² In the present study, we chose to use 100 nM capsaicin to test for modulation of TRPV1 desensitization. Previous studies using higher concentrations of capsaicin (200 nM to 1 μ M) indicate that capsaicin elicits calcium responses in a greater proportion of mouse DRG and trigeminal neurons, ranging from 30 to 70%.^{5,24,27,45,55,63}

Mouse and human sensory neurons share similar Trpv1, Grm2, and Grm3 expression and coexpression profiles

We hypothesized that the observed species differences in mGluR2/3 functional modulation of TRPV1 could be due to reduced coexpression between TRPV1 and mGlu2 and/or mGlu3 receptors in human versus mouse sensory neurons. We previously demonstrated mGluR2-immunoreactivity in human sensory neurons.²³ However, due to the lack of highly

selective mGluR3 antibodies suitable for immunohistochemistry³¹, we assessed expression of TRPV1, mGluR2, and mGluR3 mRNA transcripts (referred to as *Trpv1*, *Grm2*, and *Grm3*, respectively) in dissociated sensory neurons using RNAscope fluorescent *in situ* hybridization (FISH). The mouse DRG neurons evaluated in FISH studies ranged from 10.0 to 35.5 μm in diameter, with a mean diameter of $17.7 \pm 0.2 \mu\text{m}$ for the total population, and $20.3 \pm 0.4 \mu\text{m}$ for *Trpv1*+ neurons (Fig. 2A). In comparison, human DRG neurons ranged from 10.0 to 56.3 μm in diameter, with a mean diameter of $31.9 \pm 0.5 \mu\text{m}$ for the total population, and $33.9 \pm 0.9 \mu\text{m}$ for *TRPV1*+ neurons (Fig. 2B). The mean diameter of the total human DRG neuron population was significantly larger than that of mouse (Fig. 2C). Our findings closely resemble the size distribution of mouse DRG neurons reported previously.^{4,25,50} Previous human studies show that the average sensory neuron diameter in unfixed tissues is between approximately 40-60 μm ^{1,22,33,69,73}, further highlighting the species difference in sensory neuron size.

With respect to mRNA expression, of the total population of mouse DRG neurons, 37.2% of neurons were positive for the *Trpv1* gene transcript, 61.5% were positive for *Grm2*, and 30.9% were positive for *Grm3* (Fig. 2D). Similar transcript expression was observed in human sensory neurons, with 32.2% of all neurons positive for *TRPV1*, 52.7% positive for *GRM2*, and 29.5% positive for *GRM3* (Fig. 2E). Notably, a significantly greater percentage of total DRG neurons expressed *Grm2* compared to *Grm3* in both mouse (chi square, $\chi^2 = 74.56$, $p < 0.0001$) and human (chi square, $\chi^2 = 28.83$, $p < 0.0001$). While there was a trend towards an increased percentage of *Grm2*+ neurons in mouse compared to human DRG (chi square, Bonferroni correction, $\chi^2 = 2.229$, $p = 0.077$), there were no significant species differences in the expression of the transcripts of interest. We were also interested in whether human donors displayed differences in the percentage *GRM2*+, *GRM3*+, or *TRPV1*+ neurons, however 5 donors was not sufficient to perform these analyses on FISH data.

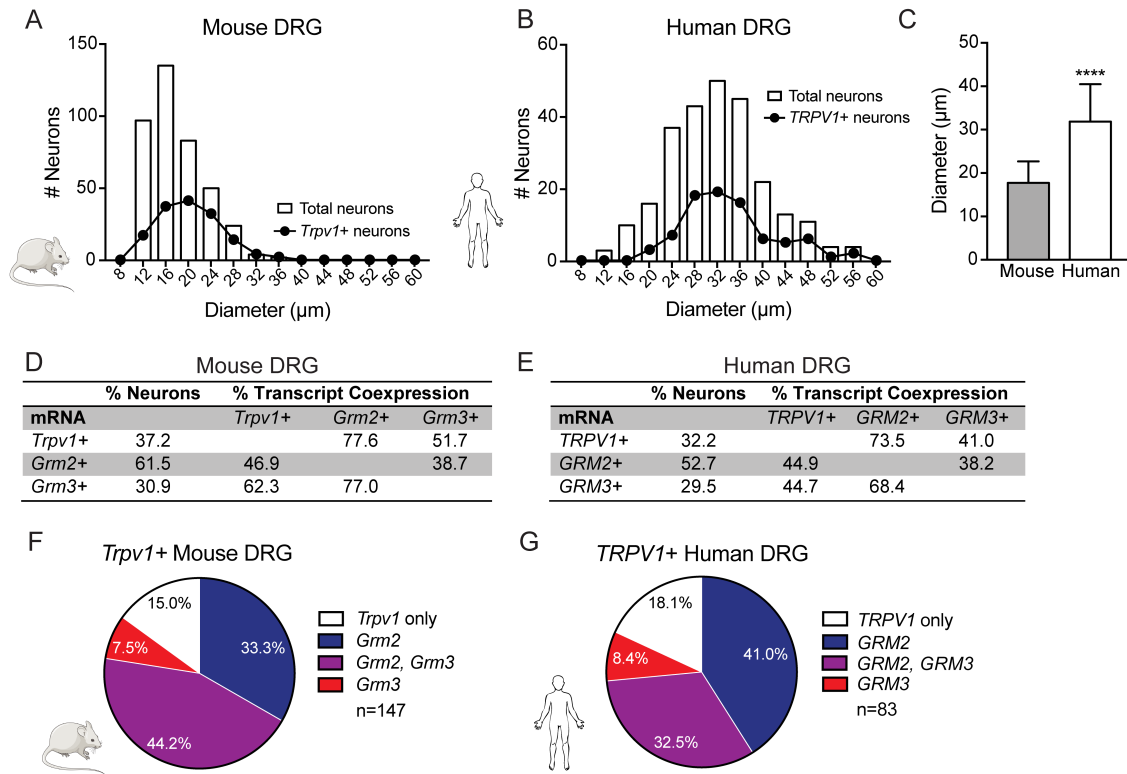


Figure 2. Expression of *Trpv1*, *Grm2*, and *Grm3* mRNA transcripts in dissociated mouse and human DRG neurons. (A) Size distribution of total and *Trpv1*+ mouse DRG neuron populations; mean diameter of total neurons: $17.7 \pm 0.2 \mu\text{m}$ ($n=395$ neurons, $N=4$ preps), mean diameter of *Trpv1*+ neurons: $20.3 \pm 0.4 \mu\text{m}$ ($n=147$ neurons). (B) Size distribution of total and *TRPV1*+ human DRG neuron populations; mean diameter of total neurons: $31.9 \pm 0.5 \mu\text{m}$ ($n=258$ neurons, $N=5$ donors), mean diameter of *TRPV1*+ neurons: $33.9 \pm 0.9 \mu\text{m}$ ($n=83$ neurons). Data are reported as mean \pm SEM (C) The mean diameter of total human DRG neurons was significantly larger than total mouse DRG neurons (unpaired t-test, **** $p<0.0001$). Data are presented as mean \pm SD. Percentage of total (D) mouse and (E) human DRG neurons that expressed *Trpv1*, *Grm2*, and *Grm3*, as well as the percentage of neurons that coexpressed one mRNA transcript with another. Pie charts showing the percentage of *Trpv1*+ (F) mouse and (G) human DRG neurons that coexpressed *Grm2*, *Grm3*, or both transcripts. No significant differences in total or *Trpv1*+ subpopulations were observed between species.

Analysis of transcript coexpression showed that the majority of *Trpv1*+ mouse DRG neurons coexpressed *Grm2*, *Grm3*, or both. To elaborate, 33.3% of *Trpv1*+ neurons coexpressed *Grm2*, 7.5% coexpressed *Grm3*, and 44.2% coexpressed both *Grm2* and *Grm3* (Fig. 2F). Of *TRPV1*+ human DRG neurons, 41.0% coexpressed *GRM2*, 8.4% coexpressed *GRM3*, and 32.5% coexpressed both *GRM2* and *GRM3* (Fig. 2G). No significant species differences were observed in the coexpression of *Trpv1* with *Grm2* and/or *Grm3* gene

transcripts. Thus, these findings suggest that the absence of mGluR2/3-induced suppression of TRPV1 sensitization in human DRG neurons cannot be explained by reduced coexpression of TRPV1 with group II mGluRs at the gene transcript level.

3.5 Discussion

Peripheral targets for pain relief are greatly desired given the centrally mediated side effects including addiction and misuse of current frontline analgesics such as opioids. In both mouse and human, activation of group II mGluRs blocks sensory neuron membrane hyperexcitability elicited by the inflammatory mediator PGE₂.²³ In the present study, we demonstrate that suppression of inflammation-induced TRPV1 sensitization represents an additional mechanism by which mGluR2/3 reduce peripheral sensitization in mouse, but not human sensory neurons. We further show equivalent colocalization of *Trpv1* with *Grm2* and *Grm3* mRNA transcripts in mouse and human DRG neurons, suggesting that disparities in coexpression do not explain species differences in the functional modulation of TRPV1 by group II mGluRs. These findings indicate that while mGluR2/3 activation decreases sensory neuron sensitization in both mouse and human, mechanisms of peripheral analgesia are not fully conserved across species.

mGluR2/3 functional differences in mouse and human sensory neurons

That the mGlu2/3 receptor agonist APDC did not suppress PGE₂-induced TRPV1 sensitization in human DRG neurons was a surprising observation. Foremost, cDNA and amino acid sequences of human and rodent mGluR2 and mGluR3 display at least 90% homology.^{29,35,42} In turn, APDC exhibits comparable potency at rodent and human group II mGluRs with respect to inhibition of stimulated cAMP responses.⁵⁷ Our recent finding that APDC decreases excitability and increases action potential threshold in PGE₂-treated sensory neurons of both species further suggests mGluR2/3 functional homology in mouse and human sensory neurons.²³ Existing behavioral and *in vitro* rodent studies strongly suggest that inhibition of cAMP-dependent TRPV1 sensitization is another mechanism by which mGluR2/3 can block sensory neuron sensitization.^{14,15,26,70} Here, we substantiate these findings by demonstrating

that APDC blocks PGE₂-induced TRPV1 sensitization in C57BL/6J mice. However, this observation did not translate in human sensory neurons despite the apparent similarities of mGluR2/3 function between species.

There are multiple potential explanations for the lack of translation of mGluR2/3 functional modulation of TRPV1 from mouse to human. For example, in addition to PKA, PGE₂-induced intracellular signaling cascades can activate other kinases known to sensitize TRPV1, including PKC and c-Src kinase.^{34,48,49,66} Thus, while we observed PGE₂-induced sensitization of capsaicin responses in both mouse and human sensory neurons, it is possible that in contrast to mouse, PGE₂-induced TRPV1 sensitization in human occurs via a predominantly PKA-independent pathway that is not influenced by mGlu2/3 receptor activation. Further, there is evidence that postsynaptic group II mGluRs can also activate PKC in rodent hippocampal and cortical neurons.^{56,62} It is therefore possible that mGluR2/3 may activate intracellular signaling messengers such as PKC that sensitize TRPV1 to different extents in mouse and human sensory neurons. Further investigation of the intracellular mechanisms that 1) underlie PGE₂-induced sensitization and 2) are initiated by mGluR2/3 in human sensory neurons is therefore needed.

Another important consideration is that while we observed equivalent coexpression of *Grm2* and *Grm3* gene transcripts in *Trpv1*⁺ mouse and human DRG neurons, whether equivalent coexpression of TRPV1 and mGluR2/3 also extends to the protein level remains unclear. For instance, it is possible that species differences exist in the regulation of translation, post-translational modifications, and subcellular compartmentalization of TRPV1 and mGlu2/3 receptors. TRPV1-immunoreactivity has been demonstrated in human DRG neurons, peripheral nerves, and intraepidermal nerve fibers.^{1,2,28,33,40} However, until selective mGluR2 and mGluR3 antibodies suitable for immunohistochemistry are generated, our ability to evaluate the coexpression and subcellular localization of group II mGluRs with TRPV1 remains limited.

Importantly, although mGlu2/3 receptor activation does not modulate sensitization of TRPV1 in human, the expression of *GRM2* and/or *GRM3* in the majority of small-diameter (< 50 μ m) *TRPV1*+ human DRG neurons suggests that these receptors are well positioned to modulate nociceptor activity by alternative mechanisms. Thus, mGluR2/3 remain putative human peripheral analgesic targets.

Sensory neuron expression of Grm2, Grm3, and Trpv1

Existing immunohistochemical analyses of rodent DRG neurons demonstrate high colocalization of group II mGlu receptors with TRPV1, with 93% of TRPV1-positive neurons expressing mGluR2/3 and effectively all mGluR2/3-positive neurons expressing TRPV1.¹⁴ Here we demonstrate that the majority ($\geq 81\%$) of *Trpv1*+ mouse and human sensory neurons also express *Grm2* and *Grm3* gene transcripts. In contrast, we found that in both mouse and human, only a subset (45-62%) of either *Grm2*+ or *Grm3*+ neurons also expressed the *Trpv1* transcript. Importantly, prior immunohistochemistry studies using nonselective mGluR2/3 antibodies and RNA-seq analysis of homogenized DRG precluded analyses of which group II mGlu receptor is predominantly expressed in sensory neurons and to what extent mGluR2 and mGluR3 are coexpressed within the same neurons. We show for the first time that *Grm2* is more highly expressed than *Grm3* in mouse and human sensory neurons. Further, while *Grm2* and *Grm3* are coexpressed in a subset (38-77%) of mouse and human sensory neurons, the transcripts are also expressed individually. These findings suggest that mGluR2 may play a larger role in modulating nociceptor excitability than mGluR3. Indeed, our previous behavioral studies demonstrate that the analgesic efficacy of group II mGlu receptor agonists persists in mGluR3^{-/-}, but not mGluR2^{-/-} mice, suggesting a greater role for mGluR2 than mGluR3 in pain regulation.⁷² As repeated dosing with group II mGlu receptor agonists causes analgesic tolerance in rodents^{20,37}, alternative strategies to reinforce endogenous activation of mGluR2 may be

required to effectively target group II mGlu receptors for clinical pain relief. Our results suggest that recently developed mGluR2-specific positive allosteric modulators may be promising agents for blockade of peripheral sensitization.^{3,30}

Distinctions between human and rodent sensory neurons

In addition to differences in mGlu2/3 receptor function, we demonstrate that human sensory neurons possess distinct fundamental properties including increased diameter and an increased percentage of capsaicin-responsive neurons compared to mouse. Using calcium imaging, we found that 33.2% of human DRG neurons responded to 100 nM capsaicin. However, it is possible that a larger proportion of human sensory neurons expresses TRPV1 and/or responds to capsaicin. For instance, previous immunohistochemical analysis of human DRG showed that roughly 55% of all DRG neurons were TRPV1-immunoreactive¹. Further, in calcium imaging experiments, ~60% of ganglionectomized DRG neurons removed due to chronic intractable pain responded to 100 nM capsaicin.^{8,9} These findings suggest that an even greater species difference in capsaicin-responsive neurons may exist between mice and humans than we report here. Of course, variability in experimental conditions or donor demographics, genetic diversity, and pain-related health conditions, could underlie differences in TRPV1 expression across human studies. For this reason, developing an extensive donor tissue bank to investigate DRG neuron gene and protein expression as well as to further characterize sensory neuron subpopulations will allow for more complete comparisons between species, and perhaps more interestingly, among donor subpopulations.

Nevertheless, while only a small number of comparative studies of rodent and human sensory neurons have been conducted, it is becoming increasingly clear that species differences exist in gene expression, ion channel properties, and action potential firing patterns.^{18,32,54,73,74} Therefore, human sensory neurons represent a vital tool for improving our

understanding of human nociceptor physiology under both normal and pathological conditions. Further, using human sensory neurons to assess the validity of putative analgesic targets identified in rodents may lead to increased translational success of preclinical findings.

3.6 Acknowledgements

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3.7 Author Contributions

Designed the experiments: TDS, RWG. Cultured human DRG neurons: TDS, MVV, MYP, LAM.

Performed the experiments: TDS. Analyzed the data: TDS, DAAB.

Wrote the manuscript: TDS.

3.8 References

1. Anand U, Otto WR, Casula MA, Day NC, Davis JB, Bountra C, Birch R, Anand P: The effect of neurotrophic factors on morphology, TRPV1 expression and capsaicin responses of cultured human DRG sensory neurons. *Neurosci Lett* 399:51–6, 2006.
2. Anand U, Yiangou Y, Sinisi M, Fox M, MacQuillan A, Quick T, Korchev YE, Bountra C, McCarthy T, Anand P: Mechanisms underlying clinical efficacy of Angiotensin II type 2 receptor (AT2R) antagonist EMA401 in neuropathic pain: clinical tissue and in vitro studies. *Mol Pain* 11:38, 2015.
3. Asseri KA, Puil E, Schwarz SKW, MacLeod BA: Group II metabotropic glutamate receptor antagonism prevents the antiallodynic effects of R-isovaline. *Neuroscience* 293:151–6, 2015.
4. Barabas ME, Kossyrev EA, Stucky CL: TRPA1 Is Functionally Expressed Primarily by IB4-Binding, Non-Peptidergic Mouse and Rat Sensory Neurons. *PLoS One* 7:1–12, 2012.
5. Barabas ME, Stucky CL: TRPV1, but not TRPA1, in primary sensory neurons contributes to cutaneous incision-mediated hypersensitivity. *Mol Pain* 9:9, 2013.
6. Le Bars D, Gozariu M, Cadden SW: Animal models of nociception. *Pharmacol Rev* 53:597–652, 2001.
7. Bates D, Mächler M, Bolker B, Walker S: Fitting Linear Mixed-Effects Models using lme4. *J Stat Softw* 67:1–48, 2014.
8. Baumann TK, Burchiel KJ, Ingram SL, Martenson ME: Responses of adult human dorsal root ganglion neurons in culture to capsaicin and low pH. *Pain* 65:31–8, 1996.
9. Baumann TK, Chaudhary P, Martenson ME: Background potassium channel block and TRPV1 activation contribute to proton depolarization of sensory neurons from humans with neuropathic pain. *Eur J Neurosci* 19:1343–51, 2004.
10. Bhawe G, Zhu W, Wang H, Brasier D., Oxford GS, Gereau RW: cAMP-Dependent Protein Kinase Regulates Desensitization of the Capsaicin Receptor (VR1) by Direct Phosphorylation. *Neuron* 35:721–31, 2002.
11. Boye Larsen D, Ingemann Kristensen G, Panchalingam V, Laursen JC, Nørgaard Poulsen J, Skallerup Andersen M, Kandiah A, Gazerani P: Investigating the expression of metabotropic glutamate receptors in trigeminal ganglion neurons and satellite glial cells: implications for craniofacial pain. *J Recept Signal Transduct Res* 34:1–9, 2014.
12. Carlton S, Hargett G: Colocalization of Metabotropic Glutamate Receptors in Rat Dorsal Root Ganglion Cells. *J Comp Neurol* 501:780–9, 2007.
13. Carlton S, Hargett G, Coggeshall R: Localization of metabotropic glutamate receptors 2/3 on primary afferent axons in the rat. *Neuroscience* 105:957–69, 2001.

14. Carlton SM, Du J, Zhou S: Group II metabotropic glutamate receptor activation on peripheral nociceptors modulates TRPV1 function. *Brain Res* 1248:86–95, 2009.
15. Carlton SM, Zhou S, Govea R, Du J: Group II/III Metabotropic Glutamate Receptors Exert Endogenous Activity-Dependent Modulation of TRPV1 Receptors on Peripheral Nociceptors. *J Neurosci* 31:12727–37, 2011.
16. Caterina MJ, Leffler A, Malmberg AB, Martin W, Trafton J, Petersen-Zeitz K, Koltzenburg M, Basbaum AI, Julius D: Impaired Nociception and Pain Sensation in Mice Lacking the Capsaicin Receptor. *Science* 288:306–13, 2000.
17. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D: The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816–24, 1997.
18. Chang W, Berta T, Kim YH, Lee S, Lee SY, Ji RR: Expression and Role of Voltage-Gated Sodium Channels in Human Dorsal Root Ganglion Neurons with Special Focus on Nav1.7, Species Differences, and Regulation by Paclitaxel. *Neurosci Bull* 1–9, 2017.
19. Chiechio S: Modulation of Chronic Pain by Metabotropic Glutamate Receptors. *Adv Pharmacol* 75:63–89, 2016.
20. Chiechio S, Zammataro M, Morales ME, Busceti CL, Drago F, Gereau RW, Copani A, Nicoletti F: Epigenetic Modulation of mGlu2 Receptors by Histone Deacetylase Inhibitors in the Treatment of Inflammatory Pain. *Mol Pharmacol* 75:1014–20, 2009.
21. Conn PJ, Pin JP: Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol* 37:205–37, 1997.
22. Davidson S, Copits BA, Zhang J, Page G, Ghetti A, Gereau RW: Human sensory neurons: Membrane properties and sensitization by inflammatory mediators. *Pain* 155:1861–70, 2014.
23. Davidson S, Golden JP, Copits BA, Ray PR, Vogt SK, Price TJ, Schmidt RE, Ghetti A, Gereau RW: Group 2 mGluRs suppress hyperexcitability in mouse and human nociceptors. *Pain* 157:2081–8, 2016.
24. Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA: Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 405:183–7, 2000.
25. Dirajlal S, Pauers LE, Stucky CL: Differential response properties of IB(4)-positive and -negative unmyelinated sensory neurons to protons and capsaicin. *J Neurophysiol* 89:513–24, 2003.
26. Du J, Zhou S, Carlton SM: Group II metabotropic glutamate receptor activation attenuates peripheral sensitization in inflammatory states. *Neuroscience* 154:754–66, 2008.

27. Elitt CM, Malin S a, Koerber HR, Davis BM, Albers KM: Overexpression of artemin in the tongue increases expression of TRPV1 and TRPA1 in trigeminal afferents and causes oral sensitivity to capsaicin and mustard oil. *Brain Res* 1230:80–90, 2008.
28. Facer P, Casula MA, Smith GD, Benham CD, Chessell IP, Bountra C, Sinisi M, Birch R, Anand P: Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4 and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy. *BMC Neurol* 7:11, 2007.
29. Flor PJ, Lindauer K, Püttner I, Rüegg D, Lukic S, Knöpfel T, Kuhn R: Molecular Cloning, Functional Expression and Pharmacological Characterization of the Human Metabotropic Glutamate Receptor Type 2. *Eur J Neurosci* 7:622–9, 1995.
30. Galici R, Jones C, Hemstapat K: Biphenyl-indanone A, a positive allosteric modulator of the metabotropic glutamate receptor subtype 2, has antipsychotic-and anxiolytic-like effects in mice. *J Pharmacol Exp Ther* 318:173–85, 2006.
31. García-Bea A, Walker MA, Hyde TM, Kleinman JE, Harrison PJ, Lane TA: Metabotropic glutamate receptor 3 (mGlu3; mGluR3; GRM3) in schizophrenia: Antibody characterisation and a semi-quantitative western blot study. *Schizophr Res* 177:18–27, 2016.
32. Han C, Estacion M, Huang J, Vasylyev D V, Zhao P, Dib-Hajj S, Waxman SG: Human Nav1.8: enhanced persistent and ramp currents contribute to distinct firing properties of human DRG neurons. *J Neurophysiol* 113:3172–85, 2015.
33. Han Q, Kim YH, Wang X, Liu D, Zhang ZJ, Bey AL, Lay M, Chang W, Berta T, Zhang Y, Jiang YH, Ji RR: SHANK3 Deficiency Impairs Heat Hyperalgesia and TRPV1 Signaling in Primary Sensory Neurons. *Neuron* 92:1279–93, 2016.
34. Jin X, Morsy N, Winston J, Pasricha PJ, Garrett K, Akbarali HI: Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase. *Am J Physiol Cell Physiol* 287:C558–63, 2004.
35. Johnson M, Schoepp DD: Group II Metabotropic Glutamate Receptors (mGlu2 and mGlu3). Gereau RW, Swanson G, editors. *The Glutamate Receptors*. 2008.
36. Johnson MP, Muhlhauser MA, Nisenbaum ES, Simmons RMA, Forster BM, Knopp KL, Yang L, Morrow D, Li DL, Kennedy JD, Swanson S, Monn JA: Broad spectrum efficacy with LY2969822, an oral prodrug of metabotropic glutamate 2/3 receptor agonist LY2934747, in rodent pain models. *Br J Pharmacol* 174:822–35, 2017.
37. Jones CK, Eberle EL, Peters SC, Monn J a, Shannon HE: Analgesic effects of the selective group II (mGlu2/3) metabotropic glutamate receptor agonists LY379268 and LY389795 in persistent and inflammatory pain models after acute and repeated dosing. *Neuropharmacology* 49:206–18, 2005.
38. Kissin I: The development of new analgesics over the past 50 years: A lack of real breakthrough drugs. *Anesth Analg* 110:780–9, 2010.

39. Kolber BJ: mGluRs Head to Toe in Pain. *Prog Mol Biol Transl Sci* 131:281–324, 2015.
40. Li Y, Adamek P, Zhang H, Tatsui CE, Rhines LD, Mrozkova P, Li Q, Kosturakis AK, Cassidy RM, Harrison DS, Cata JP, Sapire K, Zhang H, Kennamer-Chapman RM, Jawad AB, Ghatti A, Yan J, Palecek J, Dougherty PM: The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4. *J Neurosci* 35:13487–500, 2015.
41. Lopshire JC, Nicol GD: The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies. *J Neurosci* 18:6081–92, 1998.
42. Makoff A, Lelchuk R, Oxer M, Harrington K, Emson P: Molecular characterization and localization of human metabotropic glutamate receptor type 4 1. *Mol Brain Res* 37:239–48, 1996.
43. Mao J: Current challenges in translational pain research. *Trends Pharmacol Sci* 33:568–73, 2012.
44. Meves H: The action of prostaglandins on ion channels. *Curr Neuropharmacol* 4:41–57, 2006.
45. Mohammed ZA, Doran C, Grundy D, Nassar MA: Veratridine produces distinct calcium response profiles in mouse Dorsal Root Ganglia neurons. *Sci Rep* 7:45221, 2017.
46. Mohapatra DP, Nau C: Desensitization of Capsaicin-activated Currents in the Vanilloid Receptor TRPV1 Is Decreased by the Cyclic AMP-dependent Protein Kinase Pathway. *J Biol Chem* 278:50080–90, 2003.
47. Moore A, Derry S, Eccleston C, Kalso E: Expect analgesic failure; pursue analgesic success. *Br Med J* 346:f2690, 2013.
48. Moriyama T, Higashi T, Togashi K, Iida T, Segi E, Sugimoto Y, Tominaga T, Narumiya S, Tominaga M: Sensitization of TRPV1 by EP 1 and IP Reveals Peripheral Nociceptive Mechanism of Prostaglandins. *Mol Pain* 1:3, 2005.
49. Numazaki M, Tominaga T, Toyooka H, Tominaga M: Direct phosphorylation of capsaicin receptor VR1 by protein kinase C ϵ and identification of two target serine residues. *J Biol Chem* 277:13375–8, 2002.
50. O'Brien DE, Alter BJ, Satomoto M, Morgan CD, Davidson S, Vogt SK, Norman ME, Gereau GB, Demaro JA, Landreth GE, Golden JP, Gereau RW: ERK2 Alone Drives Inflammatory Pain But Cooperates with ERK1 in Sensory Neuron Survival. *J Neurosci* 35:9491–507, 2015.
51. Osikowicz M, Mika J, Makuch W, Przewlocka B: Glutamate receptor ligands attenuate allodynia and hyperalgesia and potentiate morphine effects in a mouse model of neuropathic pain. *Pain* 139:117–26, 2008.
52. Petralia RS, Wang Y-X, Niedzielski AS, Wenthold RJ: The metabotropic glutamate

- receptors, MGLUR2 and MGLUR3, show unique postsynaptic, presynaptic and glial localizations. *Neuroscience* 71:949–76, 1996.
53. De Petrocellis L, Harrison S, Bisogno T, Tognetto M, Brandi I, Smith GD, Creminon C, Davis JB, Geppetti P, Di Marzo V: The vanilloid receptor (VR1)-mediated effects of anandamide are potently enhanced by the cAMP-dependent protein kinase. *J Neurochem* 77:1660–3, 2001.
 54. Price TJ, Dussor G: DRG TXome Database [Internet]. [cited 2017 Sep 12]. Available from: <https://www.utdallas.edu/bbs/painneurosciencelab/DRGtranscriptome/search.php>
 55. Ren A-J, Wang K, Zhang H, Liu A, Ma X, Liang Q, Cao D, Wood JN, He DZ, Ding Y-Q, Yuan W-J, Xie Z, Zhang WJ: ZBTB20 regulates nociception and pain sensation by modulating TRP channel expression in nociceptive sensory neurons. *Nat Commun* 5:4984, 2014.
 56. Rosenberg N, Gerber U, Ster J: Activation of Group II Metabotropic Glutamate Receptors Promotes LTP Induction at Schaffer Collateral-CA1 Pyramidal Cell Synapses by Priming NMDA Receptors. *J Neurosci* 36:11521–31, 2016.
 57. Schoepp DD, Johnson BG, Salhoff CR, Valu MJ, Desai MA, Burnett JP, Mayne NG, Monn JA: Selective inhibition of forskolin-stimulated cyclic AMP formation in rat hippocampus by a novel mGluR agonist, 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate. *Neuropharmacology* 34:843–50, 1995.
 58. Sharpe EF, Kingston AE, Lodge D, Monn JA, Headley PM: Systemic pre-treatment with a group II mGlu agonist, LY379268, reduces hyperalgesia in vivo. *Br J Pharmacol* 135:1255–62, 2002.
 59. Simmons RMA, Webster AA, Kalra AB, Iyengar S: Group II mGluR receptor agonists are effective in persistent and neuropathic pain models in rats. *Pharmacol Biochem Behav* 73:419–27, 2002.
 60. Tang FR, Sim MK: Pre- and/or post-synaptic localisation of metabotropic glutamate receptor 1a (mGluR1a) and 2/3 (mGluR2/3) in the rat spinal cord. *Neurosci Res* 34:73–8, 1999.
 61. Team RC: R: A language and environment for statistical computing. 2017.
 62. Tyszkiewicz JP, Gu Z, Wang X, Cai X, Yan Z: Group II metabotropic glutamate receptors enhance NMDA receptor currents via a protein kinase C-dependent mechanism in pyramidal neurones of rat prefrontal cortex. *J Physiol* 554:765–77, 2004.
 63. Valtcheva M V, Davidson S, Zhao C, Leitges M, Gereau RW: Protein kinase C δ mediates histamine-evoked itch and responses in pruriceptors. *Mol Pain* 11:1, 2015.
 64. Valtcheva MV, Copits BA, Davidson S, Sheahan TD, Pullen MY, McCall JG, Dikranian K, Gereau RW: Surgical extraction of human dorsal root ganglia from organ donors and preparation of primary sensory neuron cultures. *Nat Protoc* 11:1877-88, 2016.

65. Varney MA, Gereau RW: Metabotropic glutamate receptor involvement in models of acute and persistent pain: prospects for the development of novel analgesics. *Curr Drug Targets CNS Neurol Disord* 1:283–96, 2002.
66. Vellani V, Mapplebeck S, Moriondo A, Davis JB, McNaughton PA: Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. *J Physiol* 534:813–25, 2001.
67. Whiteside GT, Kennedy J: Consideration of Pharmacokinetic Pharmacodynamic Relationships in the Discovery of New Pain Drugs. Kruger L, Light A, editors. *Translational Pain Research: From Mouse to Man*. 2010.
68. Woolf CJ: Overcoming obstacles to developing new analgesics. *Nat Med* 16:1241–7, 2010.
69. Xu Z-Z, Kim YH, Bang S, Zhang Y, Berta T, Wang F, Oh SB, Ji R-R: Inhibition of mechanical allodynia in neuropathic pain by TLR5-mediated A-fiber blockade. *Nat Med* 21:1326–31, 2015.
70. Yang D, Gereau RW: Peripheral group II metabotropic glutamate receptors (mGluR2/3) regulate prostaglandin E2-mediated sensitization of capsaicin responses and thermal nociception. *J Neurosci* 22:6388–93, 2002.
71. Yang D, Gereau RW: Peripheral group II metabotropic glutamate receptors mediate endogenous anti-allodynia in inflammation. *Pain* 106:411–7, 2003.
72. Zammataro M, Chiechio S, Montana MC, Traficante A, Copani A, Nicoletti F, Gereau RW: mGlu2 metabotropic glutamate receptors restrain inflammatory pain and mediate the analgesic activity of dual mGlu2/mGlu3 receptor agonists. *Mol Pain* 7:6, 2011.
73. Zhang X, Priest BT, Belfer I, Gold MS: Voltage-gated Na⁺ currents in human dorsal root ganglion neurons. *Elife* 6:1–23, 2017.
74. Zhang XL, Lee KY, Priest BT, Belfer I, Gold MS: Inflammatory mediator-induced modulation of GABAA currents in human sensory neurons. *Neuroscience* 310:401–9, 2015.

Chapter 4

Voluntary Exercise Training: Analysis of Mice in Uninjured, Inflammatory, and Nerve-Injured Pain States

This chapter contains the manuscript:

Sheahan TD, Copits BA, Golden JP, Gereau RW, IV: Voluntary Exercise Training: Analysis of Mice in Uninjured, Inflammatory, and Nerve-Injured Pain States. PLoS ONE 10(7): e0133191, 2015. doi:10.1371/journal.pone.0133191

4.1 Abstract

Both clinical and animal studies suggest that exercise may be an effective way to manage inflammatory and neuropathic pain conditions. However, existing animal studies commonly use forced exercise paradigms that incorporate varying degrees of stress, which itself can elicit analgesia, and thus may complicate the interpretation of the effects of exercise on pain. We investigated the analgesic potential of voluntary wheel running in the formalin model of acute inflammatory pain and the spared nerve injury model of neuropathic pain in mice. In uninjured, adult C57BL/6J mice, 1 to 4 weeks of exercise training did not alter nociceptive thresholds, lumbar dorsal root ganglia neuronal excitability, or hindpaw intraepidermal innervation. Further, exercise training failed to attenuate formalin-induced spontaneous pain. Lastly, 2 weeks of exercise training was ineffective in reversing spared nerve injury-induced mechanical hypersensitivity or in improving muscle wasting or hindpaw denervation. These findings indicate that in contrast to rodent forced exercise paradigms, short durations of voluntary wheel running do not improve pain-like symptoms in mouse models of acute inflammation and peripheral nerve injury.

4.2 Introduction

Chronic pain is a debilitating condition that affects over 100 million Americans and has an annual cost of \$635 billion in the form of health care expenses and productivity loss.^{34,45} Clinical studies suggest aerobic exercise is an effective, non-invasive approach to managing ongoing inflammatory and neuropathic pain conditions, as well as musculoskeletal disorders.^{22,23,27,43,71} Similarly, exercise improves pain-like symptoms in rodent inflammatory and neuropathic pain models.^{1,5,8,18,20,21,29,37,50,63,64,66,67}

The effect of exercise on pain has been primarily evaluated using forced exercise paradigms. For example, recent studies have demonstrated that forced treadmill running following peripheral nerve injury reverses nerve injury-induced thermal and mechanical hypersensitivity in rats and mice.^{8,20,21,49,67} Extended swimming has also been shown to both attenuate the development of and reverse nerve injury-induced thermal and mechanical hypersensitivity in rodents, as well as formalin-induced spontaneous pain in rats.^{1,50,64} As acknowledged in these studies and others, forced exercise may elicit both acute and chronic stress responses that in turn can produce analgesia.^{14,16,52,56–58} Although most forced exercise studies have evaluated nociceptive thresholds after exercise-induced acute stress responses have resolved, prolonged forced exercise gives rise to chronic stress responses in some cases.^{52,56,57} Voluntary exercise may also elicit a stress response⁵⁸; however, numerous studies demonstrate anxiolytic effects of prolonged voluntary wheel running in cases of mild to moderate stress.^{7,31,32,61,62} One way to minimize potential complications of stress dependent effects on pain is to use voluntary exercise paradigms.

Voluntary wheel running has been shown to be effective in delaying decreases in muscle withdrawal thresholds and increased paw withdrawal frequency in mouse models of chronic muscle pain.⁶⁶ Similarly, in a high-fat model of prediabetic neuropathy, voluntary wheel running reverses mechanical and visceral hypersensitivity.³⁷ While these studies support that voluntary

wheel running improves pain-like behavior in rodents, reports utilizing voluntary exercise paradigms in injured and uninjured pain states are limited.

To investigate whether repeated voluntary exercise sessions alter basal nociception in rodents, we determined whether voluntary wheel running changes nociceptive thresholds, sensory neuron excitability, or skin innervation in the absence of injury. We also investigated if voluntary exercise training alters acute inflammatory pain responses as well as the hypersensitivity, muscle wasting, or skin denervation induced by the spared nerve injury (SNI) model of neuropathic pain. We report that voluntary wheel running did not alter nociceptive thresholds in uninjured mice, and was ineffective in attenuating acute inflammatory or nerve-injury induced pain.

4.3 Materials and Methods

Ethics Statement

The entire study was carried out in accordance to the guidelines of the Washington University in St. Louis Department of Comparative Medicine (DCM). The protocol was approved by the Animal Studies Committee of DCM (Protocol Number: 20130147).

Animals

Adult C57BL/6J male mice bred in house or obtained from Jackson Labs were housed and cared for in compliance with the Animal Studies Committee of Washington University in St. Louis. Mice were housed on a 12/12 hour (hr) light/dark cycle and had *ad libitum* access to food and water while in their home cages. Behavioral experiments were initiated on 7-10 week old mice. Over the course of testing, injury (if applicable), and exercise training, mice reached a maximum age of 14 weeks. At the conclusion of behavioral testing, mice were sacrificed using a rodent ketamine euthanasia cocktail.

Exercise paradigm

During exercise training, mice were placed into individual cages with low-profile wireless running wheels (Med Associates Inc.) for either 2 hr (6-8 PM) or 12 hr (7 PM-7 AM) a night, 5-6 nights a week. A sixth night of training was required to maintain exercise states throughout behavioral testing. Distance ran was monitored using software from Med Associates Inc. Control animals were placed into individual cages with a locked running wheel. During 2 hr training sessions, white noise was used to mask external noises. Animals trained overnight were provided food pellets and Hydrogels (ClearH₂O) to prevent weight loss. Home cage access to running wheels – either unlocked or locked – was not used in our studies to minimize stress

associated with the chronic social isolation of single housing.⁴⁰ When not exercising, animals were group housed in their home cages.

Exercise training was completed prior to the induction of acute inflammatory pain, which took place the afternoon following the last exercise bout. Specifically, intraplantar formalin was administered 16 hr after completion of 2 hr/night exercise sessions or 4-6 hr after completion of 12 hr/night exercise sessions. In nerve-injury studies, exercise training began 8-10 days after surgery.

Behavioral studies

All behavioral assays were completed between 8 AM and 5 PM. Briefly, for the Hargreaves, von Frey, and cold plantar assays, mice were acclimated in plexiglass boxes to the testing platform and white noise for at least 2 hr prior to testing until exploratory behavior ceased. For the hot plate and inverted screen tests, mice were acclimated to the testing room in their home cage with white noise for at least 1 hr prior to testing. For all behavioral studies, the experimenter was blinded to training groups.

Cold plantar assay

Cold sensitivity was measured as previously described.^{12,13} Mice were acclimated to either a 3/8" or 1/4" glass plate and a cold probe was made by packing finely crushed dry ice into a modified syringe 1 cm in diameter. The cold probe was applied to the glass beneath the plantar surface of the hindpaw and the time to paw withdrawal was recorded. 5 trials were conducted on each hindpaw, with 7 minutes (min) between trials on opposite paws, and 15 min between trials on the same paw. A cut off latency of 20 seconds (sec) was used to prevent tissue damage. Withdrawal latencies were determined by averaging right and left paw responses.

Hargreaves

Animals were acclimated on a glass plate held at 30°C (Model 390 Series 8, IITC Life Science Inc.). A radiant heat source was applied to the hindpaw and latency to paw withdrawal was recorded.³⁹ 5 trials were conducted on each paw, with at least 5 min between testing the opposite paw and at least 10 min between testing the same paw. To avoid tissue damage, a cut off latency of 20 sec was set. Values from both paws were averaged to determine withdrawal latency.

Hot plate

Prior to testing day, animals were individually acclimated to the hot plate chamber (Model PE34 Series 8, IITC Life Science Inc.) for 15 min. On the day of testing, one experimental trial was conducted in which each animal was placed onto a 55°C plate and the latency to response was recorded. "Response" was defined as the first act directed towards the hindpaw, which was primarily licking or shaking, or escape behavior such as jumping. A cut off latency of 20 sec was used to prevent tissue damage.

von Frey

Mice were acclimated on an elevated mesh grid and mechanical sensitivity was determined by applying von Frey filaments to the plantar hindpaw according to the up-down method as described previously.¹⁷ Three trials were conducted on each paw with at least 5 min between trials on opposite paws, and 10 min between trials on the same paw. Mechanical paw withdrawal thresholds of uninjured animals were obtained by averaging the 3 trials performed on both paws. Nerve-injured animals were tested on the lateral aspect of the hindpaw at both baseline and post-injury time points, and the average ipsilateral withdrawal thresholds were calculated.²⁵

Formalin test

For acute inflammatory pain, mice were acclimated in plexiglass boxes on a glass platform for 1 hr with white noise. Mice were briefly removed from the platform and 10 μ L of 2% formalin was injected subcutaneously into the hindpaw. Mice were video recorded for 1 hr after formalin injection and time spent licking and lifting the paw post-injection was scored in 5-min bins offline. To minimize potential acute effects of exercise on behavior^{48,65}, the formalin test was performed at least 16 hr after the final 2 hr training session, and at least 4 hr after the final overnight training session.

Inverted screen test

To evaluate muscle strength, mice were allowed to grasp onto a mesh grid. The grid was then inverted until mice were upside-down and the latency to fall recorded, using a cut off latency of 120 sec. Two trials were performed with at least 1 hr of rest allowed between trials.³

Dissociating DRG neurons

Mice were sacrificed via live decapitation and lumbar (L) dorsal root ganglia (DRG) 3-5 were dissected from control and exercise trained mice. DRG were incubated in papain (45U) for 20 min, rinsed, and incubated with collagenase (4.5 mg/200 μ L) for 20 min. DRG were triturated to dissociate neurons and filtered with a 40 μ m mesh filter. The dissociated cells were plated on poly-D-lysine/collagen coated glass coverslips and were tested 12-24 hr after plating.

Electrophysiology

Whole-cell recordings were made in current clamp on cells 20-30 μ m in diameter to increase the likelihood of recording from nociceptive neurons. Fire polished glass pipettes were pulled using a P-97 micropipette puller (Sutter Instrument Company), and open tip resistances

ranged from 2.0-7.0 M Ω . Pipettes were filled with internal recording solution consisting of (in mM): 120 K⁺ gluconate, 5 NaCl, 2 MgCl₂, 0.1 CaCl₂, 10 HEPES, 1.1 EGTA, 4 Na₂ATP, 0.4 Na₂GTP, 15 phosphocreatine; pH=7.3, 291 mOsm. While recording, cells were continuously perfused with room temperature external solution consisting of (in mM): 145 NaCl, 3 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 10 HEPES, 7 glucose, adjusted to pH 7.4 with NaOH. The series resistance of each recording was less than 20 M Ω . Patchmaster software (Heka Instruments Inc.) controlling an EPC10 USB amplifier (Heka Instruments Inc.) was used to record from neurons. Only neurons with a resting membrane potential more negative than -45 mV were included in data analysis.

Spared nerve injury model

Throughout surgery, mice were maintained under isoflurane anesthesia. A skin incision was made and the biceps femoris muscle was separated to expose the branches of the sciatic nerve. Without manipulating the sural nerve branch, the common peroneal and tibial branches were ligated with 6-0 silk suture and approximately 1 mm of nerve was removed distally.²⁵ The wound was closed in layers and mice were monitored for signs of distress and allowed to recover post-operatively on a heating pad prior to returning to their home cages. Surgical procedures were approved by the Washington University in St. Louis DCM (Protocol Number: 20130147).

Gastrocnemius weight

The gastrocnemius muscle was excised by cutting proximally from medial and lateral condyles of the femur and distally at the calcaneal tuberosity.⁷³ Tissue was promptly weighed after dissection to obtain muscle wet weight.

Immunohistochemistry and hindpaw innervation

After sacrificing animals with a rodent ketamine euthanasia cocktail, plantar hindpaw skin was excised and immersion fixed for 2-6 hr in 15% picric acid/2% paraformaldehyde at 4°C, washed in PBS, and cryoprotected in 30% sucrose. Hindpaw skin was sectioned at 30 µm perpendicular to the skin's surface. To stain hindpaw skin, the primary antibodies rabbit anti-βIII tubulin 1:1000 (Covance PRB-435P) and goat anti-rat CGRP 1:400 (AB Serotec 1720-9007) were visualized using donkey anti-rabbit Alexa-fluor 555 1:200 (Invitrogen A31572) and donkey anti-goat Alexa-fluor 488 1:350 (Invitrogen A11055), respectively. Images were captured using an upright epifluorescent microscope (Nikon 80i, CoolSnap ES camera). MetaMorph software (Molecular Devices, LLC) was used to measure dermal-epidermal border length, and labeled intraepidermal fibers were counted and averaged from 5 nonsequential sections per animal. Intraepidermal nerve fiber (IENF) density is reported as number of fibers/100 µm dermal-epidermal border.

Epidermal thickness

The vertical distance from the dermal-epidermal border to the outermost granular cell layer – visualized via DAPI staining – was measured at 5 separate points at 50 µm intervals to obtain the average thickness of each section from which IENFs were counted.

Statistical analyses

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc.) and are presented as mean ± SEM. Withdrawal latencies and thresholds are normalized to baseline values obtained prior to exercise training or SNI. Data comparing the effect of exercise training as well as formalin responses over time were analyzed with a Student's t-Test. A two-way repeated-measures (RM) analysis of variance (ANOVA) was used to analyze the effect of SNI

and exercise training on mechanical thresholds. SNI muscle strength, wet weight, innervation, and epidermal thickness were compared to uninjured controls by a one-way ANOVA. In all analyses, a Bonferroni test was used to correct for multiple comparisons.

4.4 Results

Nociceptive thresholds were unaltered in uninjured, exercise trained animals

To determine whether exercise training alters the nociceptive thresholds of uninjured mice, we gave mice access to running wheels for 2 hr a night, 5-6 nights a week, for 1-4 weeks. There was an average nightly running distance of 1.5 ± 0.1 km across cohorts. We are confident that the control and exercised animals differ solely in exercise training because during running sessions, controls experienced the same environmental enrichment and social isolation as exercised animals, but were unable to run on the locked wheel. In this regard, voluntary wheel running allows for more comprehensive control groups than forced exercise paradigms.

Nociceptive thresholds were measured in uninjured mice at least 12 hr after the conclusion of training sessions to minimize the potential short-term effects of exercise on behavior.^{48,65} Following 1, 2, or 4 weeks training, cold sensitivity thresholds of exercised mice measured by the cold plantar assay remained unchanged when compared to controls (Fig. 1A). Similarly, noxious heat withdrawal latencies were unchanged in the Hargreaves test after up to 4 weeks of exercise training (Fig. 1B). Heat sensitivity was also tested using the hot plate test, which incorporates supraspinal processing of nociceptive stimuli.^{15,60} Latency to response to a 55°C hot plate was not different between control and exercise trained mice (Fig. 1C). Lastly, exercise training did not alter mechanical withdrawal thresholds in the von Frey test (Fig. 1D).

Sensory neuron excitability was unchanged following exercise training

Increased excitability of DRG neurons has been shown to underlie hypersensitivity in a number of pain states.^{11,47,55} To determine whether exercise affects membrane and cell excitability properties in an uninjured context, whole-cell patch clamp electrophysiology was performed on dissociated L3-L5 DRG neuron cultures obtained from exercise trained and control animals within 24 hr of culturing (Fig. 2A). Recordings were performed on small diameter

neurons ranging from 20-30 μm to increase the likelihood of recording from nociceptive neurons.

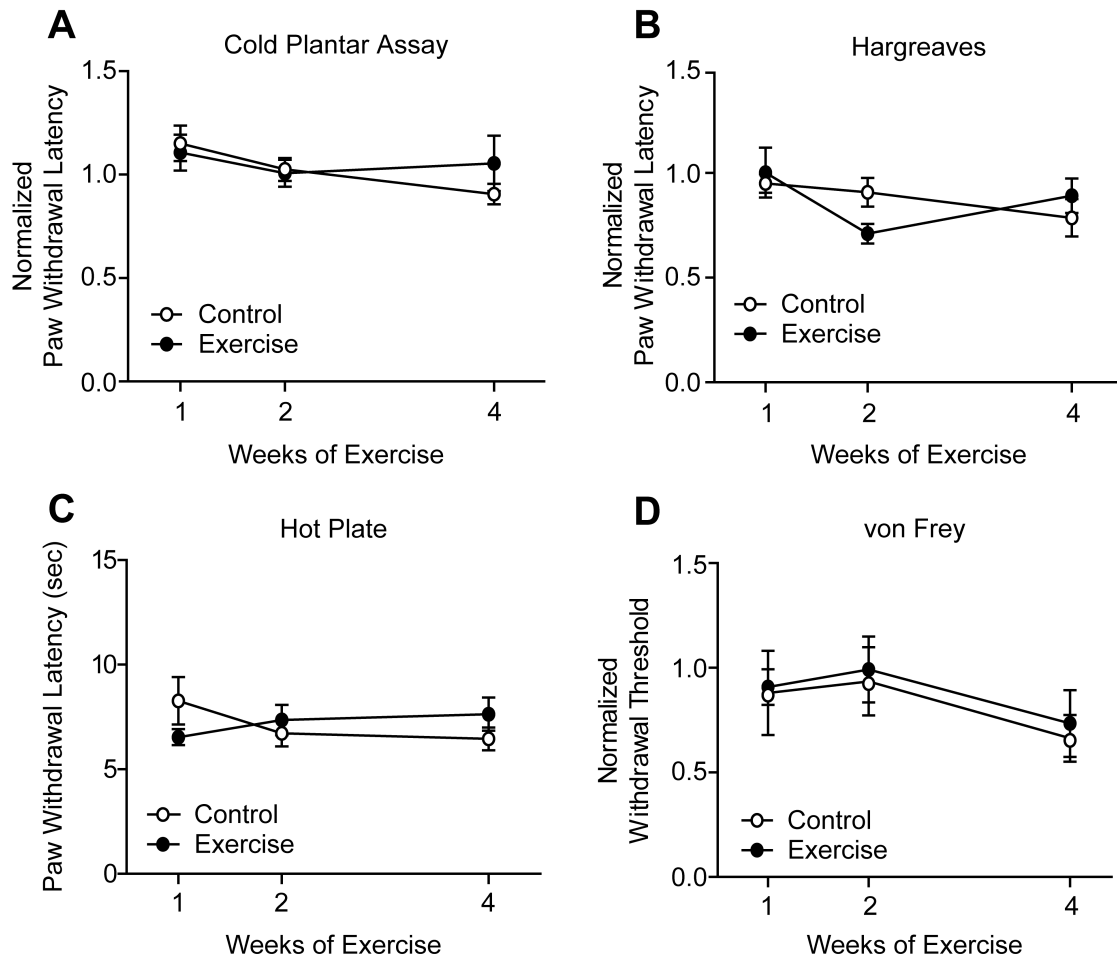


Figure 1. Exercise training did not alter thermal or mechanical sensitivity of uninjured animals. (A) Following 1, 2, or 4 weeks of exercise training, paw withdrawal latencies in the cold plantar assay remained unchanged in exercise trained versus control mice, $n=6-11$. (B, C) Withdrawal latencies to noxious heat stimuli of exercise trained and control mice were equivalent in both Hargreaves and hot plate tests, $n=9-15$ and $9-10$, respectively. (D) Mechanical withdrawal thresholds were similarly unchanged by exercise, $n=9-15$. Withdrawal latencies and thresholds are normalized to baseline values obtained prior to exercise training. Data are presented as mean \pm SEM. Student's t-Test, Bonferroni correction for multiple comparisons.

Consistent with behavioral experiments, 1-4 weeks of voluntary exercise training did not alter any of the membrane or cell excitability properties analyzed (Table 1). Resting membrane potential was unchanged in exercise trained animals as compared to controls (Fig. 2B). In current clamp, step (Fig. 2C) and ramp (Fig. 2D) current injections were used to analyze excitability properties. Rheobase, the current required to elicit an action potential, in response to

either step (Fig. 2E) or ramp (Fig. 2F) current injections were similarly unaffected by 1-4 weeks of wheel running. The number of action potentials in response to step and ramp current injections 2 and 3 times rheobase were also quantified to investigate if differences in excitability were apparent at higher stimulus intensities. Exercise training for 4 weeks did not change DRG excitability in response to these stimuli (Fig. 2G, H). Similar results were observed after 1 and 2 weeks of exercise training. Overall, DRG membrane and excitability properties were unaffected by exercise in an uninjured context.

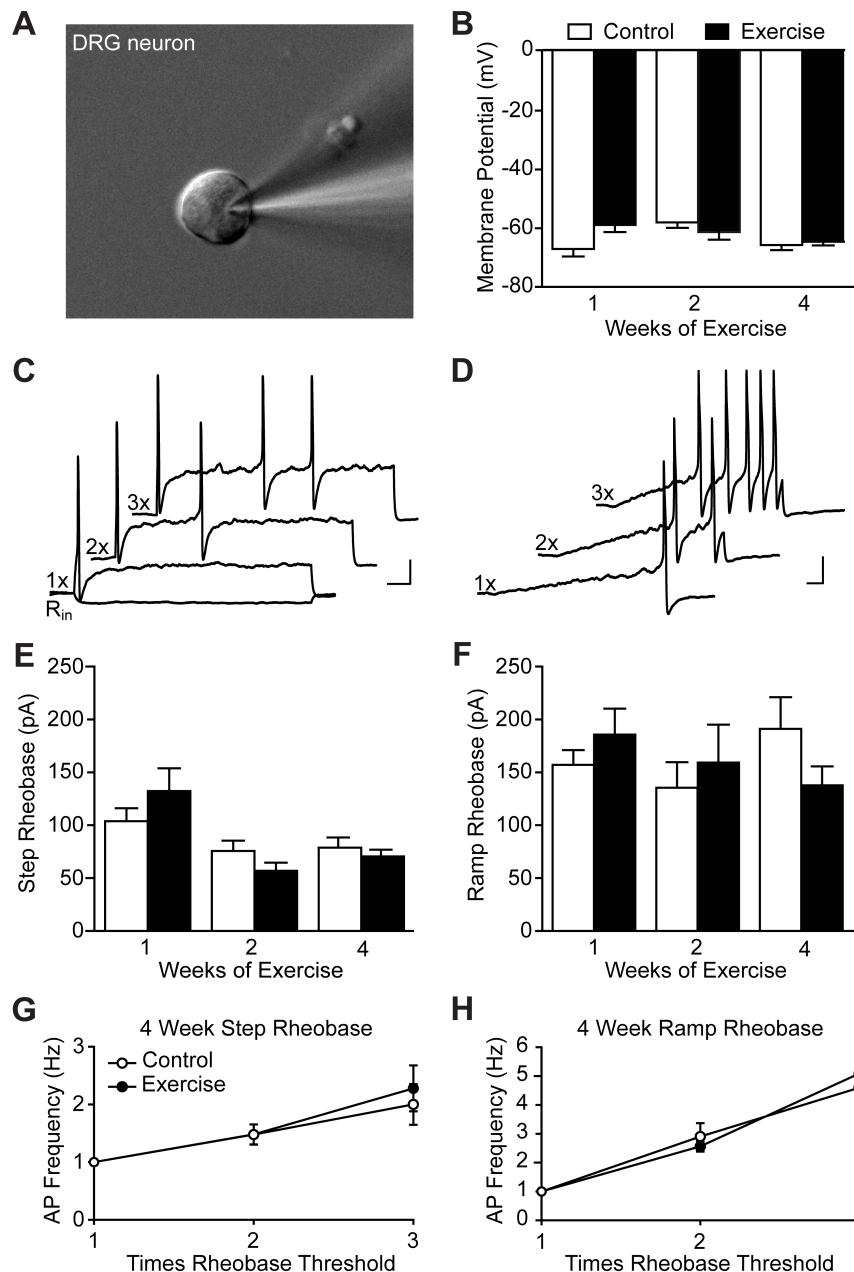


Figure 2. Exercise training did not alter DRG neuron membrane or excitability properties of uninjured animals. Whole-cell patch clamp electrophysiology was performed on small-diameter cultured lumbar DRG neurons, N=2-3 animals. **(A)** An example of a patched neuron approximately 23 μm in diameter. **(B)** Resting membrane potential was unaltered by exercise training for 1, 2 or 4 weeks, n=13-25. Representative traces of action potentials elicited by step **(C)** and ramp **(D)** current injections of 1 to 3 times rheobase. A negative stepwise current was used to determine input resistance (R_{in}). Scale bars represent 20 mV and 100 ms. Rheobase in response to both step **(E)** and ramp **(F)** current injection was also unchanged by exercise, n = 13-25 and 7-23, respectively. The number of action potentials elicited by step **(G)** and ramp **(H)** current injections 2 and 3 times rheobase was unaffected by 4 weeks of exercise training, n=22-25 and 20-23, respectively. Data are presented as mean \pm SEM. Student's t-Test, Bonferroni correction for multiple comparisons.

Table 1. Membrane and action potential parameters of lumbar DRG neurons from control and exercise trained animals

Training Duration	1 week		2 weeks		4 weeks	
Parameter	Control	Exercise	Control	Exercise	Control	Exercise
Cell Diameter, μm	25.6 \pm 0.5	25.2 \pm 0.5	23.8 \pm 0.7	24.2 \pm 0.5	24.6 \pm 0.4	24.3 \pm 0.3
Membrane Potential, mV	-67.1 \pm 2.5	-58.9 \pm 2.4	-58.0 \pm 1.9	-61.6 \pm 2.7	-65.7 \pm 1.7	-64.6 \pm 1.2
Input Resistance, M Ω	798 \pm 123	608 \pm 89	916 \pm 133	1396 \pm 309	1041 \pm 127	936 \pm 92
Capacitance, pF	18.2 \pm 1.0	20.5 \pm 1.2	17.2 \pm 1.8	18.6 \pm 2.2	17.8 \pm 1.0	17.8 \pm 1.1
Step Rheobase, pA	104 \pm 12	132 \pm 21	76 \pm 10	57 \pm 8	79 \pm 10	70 \pm 7
Step AP Threshold, mV	-22.7 \pm 2.3	-21.2 \pm 2.0	-21.0 \pm 1.8	-23.6 \pm 1.2	-22.2 \pm 1.3	-20.9 \pm 1.9
Step AP Peak, mV	49.6 \pm 1.2	49.2 \pm 1.0	45.0 \pm 1.2	47.4 \pm 2.0	50.0 \pm 1.1	50.4 \pm 0.5
Ramp Rheobase, pA	157 \pm 14	186 \pm 24	136 \pm 24	159 \pm 36	191 \pm 30	138 \pm 18
Ramp Threshold, mV	-11.5 \pm 1.6	-14.5 \pm 2.2	-12.3 \pm 2.9	-16.2 \pm 2.3	-13.6 \pm 1.6	-13.5 \pm 1.6
Ramp AP Peak, mV	51.7 \pm 1.4	51.3 \pm 1.2	44.3 \pm 1.6	47.3 \pm 2.4	51.3 \pm 1.2	50.8 \pm 0.6

AP, action potential. Data are presented as mean \pm SEM. N = 2–3 animals, n = 7–25 cells. Student's t-Test, Bonferroni correction for multiple comparisons.

Acute inflammatory pain responses were not changed by exercise training

Forced exercise has previously been shown to reduce formalin-induced spontaneous pain behavior.⁵⁰ We asked whether voluntary exercise (pre-training) similarly attenuates acute inflammatory pain responses. Formalin was administered approximately 16 hr after the final exercise session and spontaneous nocifensive behavior was recorded. Mice that received wheel access for 2 hr/night for 1–4 weeks displayed the same biphasic response to formalin as controls (Fig. 3A-C). To examine whether an increased dose of wheel running could affect acute inflammatory pain responses, exercise training was extended to 12 hr/night. On average mice ran 9.9 \pm 1.2 km during 12 hr wheel running sessions. This distance is comparable to³⁷ if not greater than^{31,66} distances reported by studies in which mice received home cage wheel access. Again, exercise for 2 weeks prior to testing did not influence formalin-induced spontaneous behavior (Fig. 3D). Formalin injections took place 4–6 hr after completion of the final 12 hr/night

exercise session. For each duration and dose of exercise tested, the cumulative time spent licking and lifting the hindpaw in the first (0-10 min) and second (11-60 min) phases of the formalin test were not different between exercise trained and control mice (Table 2).

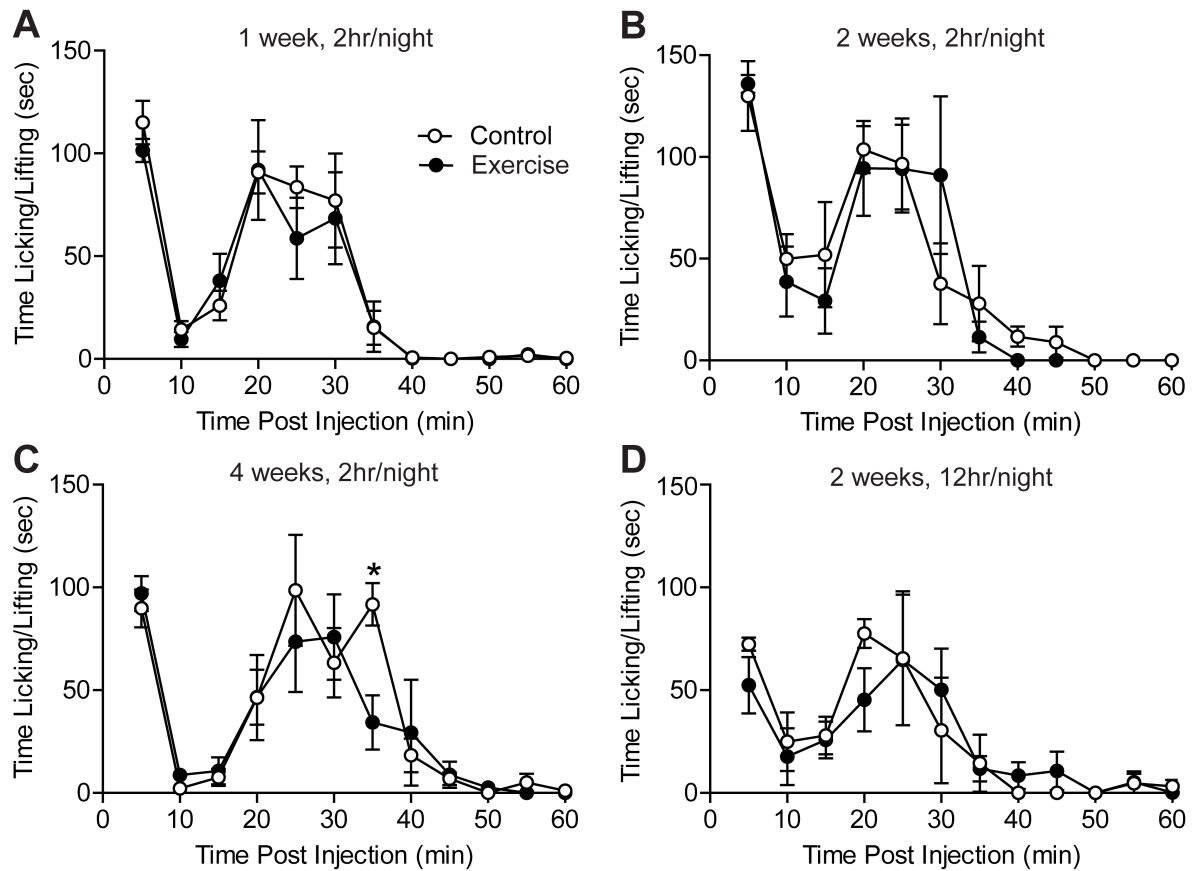


Figure 3. Exercise training did not attenuate nocifensive responses to acute inflammatory pain. (A-C) Wheel access for either 2 hr/night for 1 to 4 weeks, $n=5$ or (D) 12 hr/night for 2 weeks, $n=4-6$ prior to the formalin test did not reduce time spent licking or lifting the hindpaw during the first 60 minutes following intraplantar injection of 2% formalin. Data are presented as mean \pm SEM. Student's t-Test, Bonferroni correction for multiple comparisons.

Table 2. Cumulative time spent licking and lifting the hindpaw during Phase I and II of the formalin test.

Training	Phase I (0–10 min)		Phase II (11–60 min)	
	Control	Exercise	Control	Exercise
1 week, 2hr/night	129.3 \pm 10.1s	110.9 \pm 5.6s	296.2 \pm 20.1s	275.4 \pm 33.9s
2 weeks, 2hr/night	179.9 \pm 17.9s	174.6 \pm 16.8s	338.6 \pm 47.1s	320.4 \pm 30.7s
4 weeks, 2hr/night	91.9 \pm 9.8s	105.7 \pm 10.8s	338.9 \pm 52.3s	281.8 \pm 42.3s
2 weeks, 12hr/night	97.3 \pm 15.5s	70.1 \pm 23.0s	223.7 \pm 49.8s	223.3 \pm 46.9s

Data are presented as mean \pm SEM, $n = 4-6$. Student's t-Test, Bonferroni correction for multiple comparisons.

Exercise training did not improve mechanical hypersensitivity or muscle wasting following peripheral nerve injury

The SNI rodent model of neuropathic pain produces prolonged and robust mechanical hypersensitivity in the ipsilateral hindpaw.^{25,30} Forced exercise has been shown to reverse mechanical hypersensitivity in other peripheral nerve injury models including chronic constriction injury and spinal nerve ligation.^{20,21,67} We asked whether voluntary wheel running could similarly reverse mechanical hypersensitivity caused by SNI.

Unilateral SNI produced a significant reduction in mechanical withdrawal thresholds relative to baseline (Two-way RM ANOVA: SNI, 2 hr/night $p<0.0001$, SNI, 12 hr/night $p<0.0001$) (Fig. 4). Once the development of mechanical hypersensitivity was verified on post-operative days (POD) 3 or 4 and 6 or 8, we began exercise training mice on POD 8-10 to determine if voluntary exercise could reverse nerve injury-induced mechanical hypersensitivity. After each week of

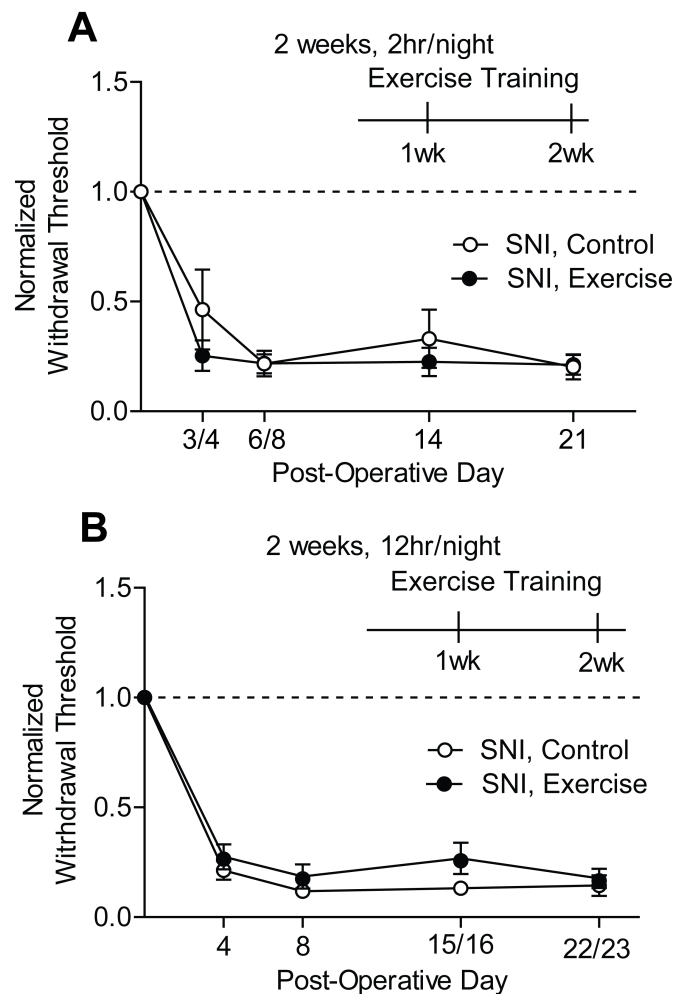


Figure 4. Exercise training did not improve SNI-induced mechanical hypersensitivity. (A, B) SNI gave rise to a significant reduction in mechanical withdrawal thresholds of the ipsilateral hindpaw in both SNI, control and SNI, exercise groups on POD 3/4 and 6/8 relative to baseline values (dotted line) (SNI, 2 hr/night $p<0.0001$, SNI, 12 hr/night $p<0.0001$, $n=7-8$ and $5-7$, respectively). When initiated 8 to 10 days post-op, 2 weeks of exercise training for either 2 hr (A) or 12hr (B) per night did not reverse SNI-induced mechanical hypersensitivity. Mechanical withdrawal thresholds were tested at one-week increments after exercise began. The one-week time point was on POD 14,15, or 16. The two-week time point was on POD 21, 22, or 23. Data are presented as mean \pm SEM. Two-way RM ANOVA, Bonferroni correction for multiple comparisons.

training, mechanical withdrawal thresholds were tested within 3 (12 hr/night group) to 12 hr (2 hr/night group) of the last training session. The one and two week post-training von Frey testing time points ranged from POD 14-16 and POD 21-23 across cohorts, respectively. Neither 2 hr/night (Fig. 4A) nor 12 hr/night (Fig. 4B) of exercise training for 2 weeks improved SNI-induced mechanical hypersensitivity.

The selection of a voluntary wheel running paradigm in the current study gave rise to the concern that nerve-injured mice would exercise much less, if at all, compared to uninjured mice. Such activity impairment would decrease the likelihood of observing exercise-induced improvements following SNI. However, quantification of weekly running distances indicates that SNI mice ran equivalent distances to uninjured animals each week of training when sessions were either 2 or 12 hr/night (Table 3). These findings eliminate concerns about significant differences in exercise dose between injured and uninjured mice.

Table 3. SNI did not depress weekly voluntary wheel running distances.

Training Session	2 hr/night		12 hr/night	
	Week 1	Week 2	Week 1	Week 2
Uninjured	7.3 ± 0.5km	10.6 ± 0.9km	35.6 ± 5.2km	64.5 ± 6.5km
SNI	5.3 ± 0.3km	8.8 ± 0.5km	24.8 ± 2.4km	50.4 ± 6.2km

Data are presented as mean ± SEM. 2hr/night n = 8–34, 12hr/night n = 6–8.

Student's t-Test, Bonferroni correction for multiple comparisons.

In addition to hypersensitivity, sciatic nerve injury is associated with denervation, atrophy, and in turn muscle weakness.^{10,73} Exercise following sciatic nerve injury can enhance muscle reinnervation, as well as grip strength and hindlimb motor function.^{8,9,21,44} We tested if muscle strength is improved in SNI mice that were exercise trained for 12 hr/night for 2 weeks using the inverted screen test.³ In trial one, both SNI, control and SNI, exercised mice had a shorter latency to fall than uninjured control mice that did not reach statistical significance (Fig. 5A). In trial two, latency to fall of both SNI groups was significantly shorter than uninjured, control mice (One-way ANOVA: p=0.0002). While mean latency to fall of SNI, exercise animals

was longer than that of SNI, control animals, the difference is not statistically significant. Interestingly, neither SNI group exhibited a training effect between trials, which may be due to muscle atrophy. We found that SNI mice did not use the injured hindpaw to grip the screen. Consistent with this possibility, we did not find that muscle strength was improved following exercise training in uninjured animals. However, we used a cutoff latency of 120 sec, which may have masked potential effects of exercise on inverted screen performance in longer experimental trials. A previous study found that 8 weeks, but not 5 days, of home cage wheel access increases both forepaw and hindpaw grip strength in mice.⁶⁶

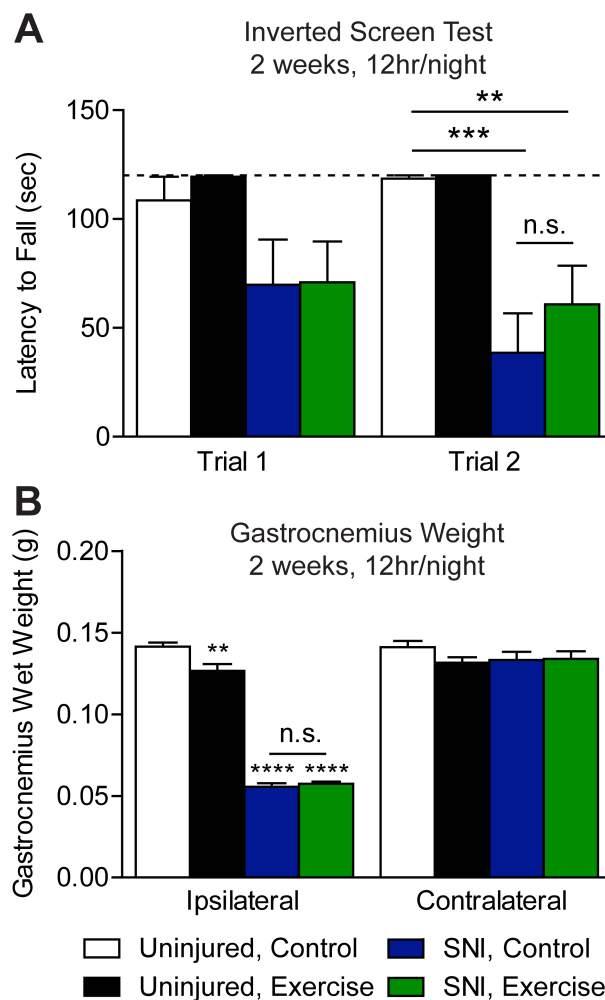


Figure 5. Exercise training did not reduce nerve injury-induced muscle wasting. (A) The inverted screen test of muscle strength was performed on mice 3 weeks (POD 21/22) after injury with a cut off latency of 120 sec (dotted line). SNI mice had a shorter latency to fall than uninjured mice in Trial 2 only ($p=0.0002$). Despite exercise training for 12 hr/night for 9 nights, latency to fall of SNI, exercise mice was not improved compared to SNI, control mice, $n=6-8$. **(B)** By POD 25 SNI gave rise to a robust reduction in ipsilateral gastrocnemius muscle wet weight ($p<0.0001$), which was unchanged by exercise, $n=6-8$. Compared to uninjured controls, gastrocnemius muscle wet weight of uninjured, exercise mice was significantly reduced in the ipsilateral ($p<0.01$), but not the contralateral hindlimb. Data are presented as mean \pm SEM. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, One-way ANOVA, Bonferroni correction for multiple comparisons.

To test whether voluntary wheel running improved muscle atrophy following SNI, we measured gastrocnemius wet weight. On POD 25, we observed a significant reduction in ipsilateral gastrocnemius muscle wet weight in SNI mice compared to uninjured controls (One-way ANOVA: $p < 0.0001$), while no change was observed in the contralateral muscle (Fig. 5B). Wheel running for 2 weeks, 12 hr/night did not reduce gastrocnemius muscle wasting. We observed a significant reduction in wet weight of the ipsilateral (One-way ANOVA: $p < 0.01$), but not contralateral gastrocnemius, of uninjured, exercised mice compared to uninjured controls (Fig. 5B). A previous report indicates gastrocnemius wet weight is unchanged by forced or voluntary exercise in an uninjured context.⁵²

Hindpaw epidermal innervation and thickness were not changed by exercise training in uninjured or nerve-injured animals

Voluntary wheel running has been shown to prevent increases in peptidergic IENF fiber density in a mouse model of prediabetic neuropathy.³⁸ We investigated whether wheel running changes IENF density or fiber phenotypic distribution in uninjured and SNI animals.

To determine if exercise training alters total IENF density, peripheral nerve fibers in hindpaw skin sections were identified by the neuron-specific marker β III tubulin (Fig. 6A, red). Hindpaw innervation was further examined using a CGRP antibody as a marker of peptidergic fibers (Fig. 6B, red). IENF were defined as those fibers crossing the dermal-epidermal border, which was visualized with DAPI (Fig. 6A, B, blue). Quantification of total IENF density in hindpaw skin indicated no difference between control mice and mice exercise trained for 1-4 weeks, 2 hr/night (Fig. 6C). The typically sparse CGRP+ IENF density relative to total IENF density was similarly unchanged by exercise at any time point (Fig. 6D).

SNI causes marked denervation of the center aspect of plantar hindpaw skin within two weeks that begins to recover about 5 weeks after injury.³³ To determine if exercise reduces this

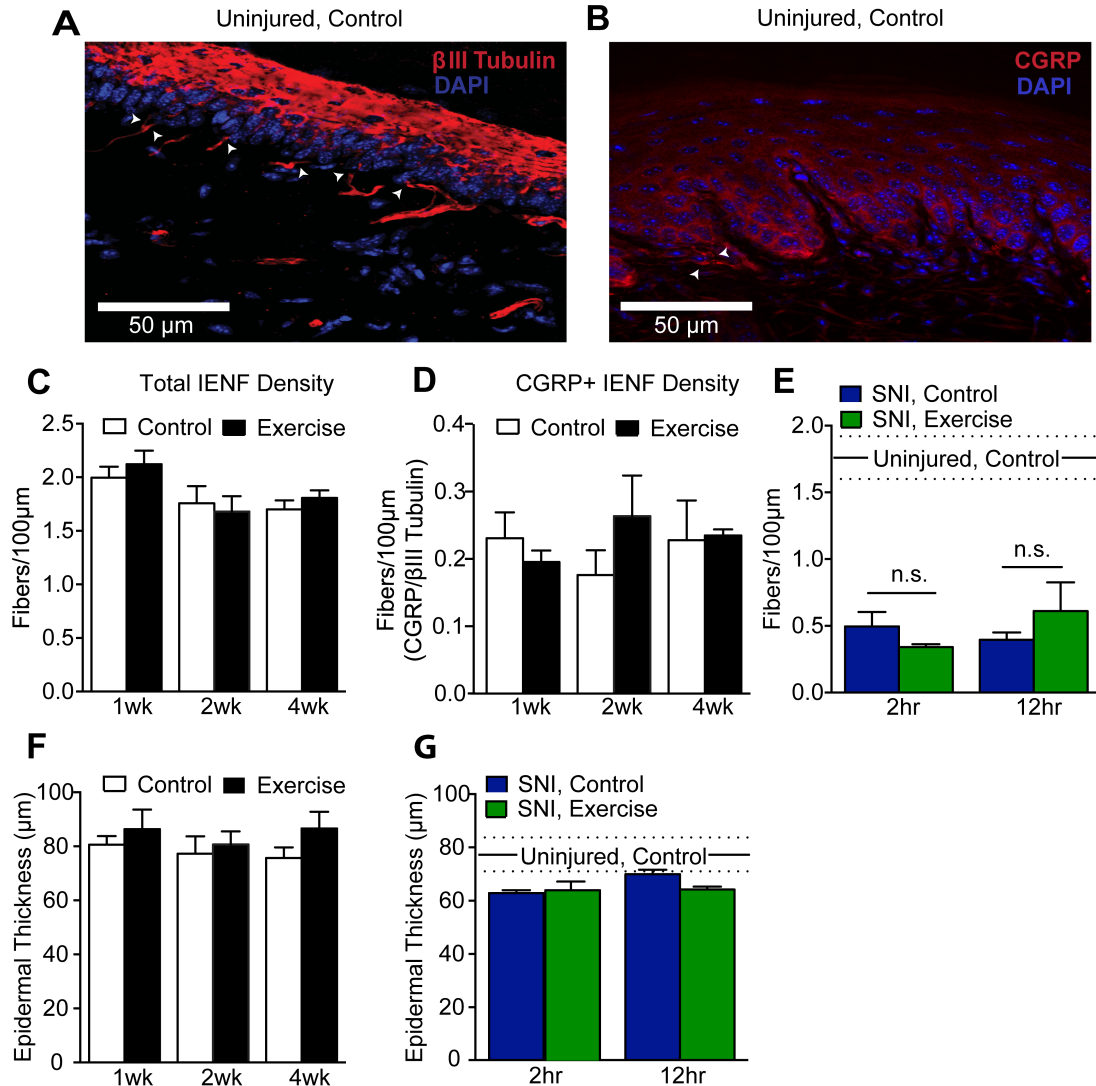


Figure 6. Exercise training did not alter hindpaw epidermal innervation or thickness in uninjured or SNI animals. (A, B) 30 μ m sections of plantar hindpaw skin were stained with β III tubulin (red) and CGRP (red) to quantify total and peptidergic IENF density, respectively. Arrowheads indicate IENF. DAPI (blue) staining was used to visualize the granular cell layer of the epidermis and measure epidermal thickness. (C, D) Both total (β III tubulin+) and peptidergic (CGRP+) IENF density were unchanged by 1-4 weeks of exercise training in uninjured mice, n=4-6. Student's t-Test. (E) 25 days after injury, a significant reduction in total (β III tubulin+) IENF density was observed in SNI, control mice compared to uninjured controls ($p < 0.0001$, n=4). Exercise training for either 2 or 12 hr per night for 2 weeks did not improve hindpaw epidermal innervation following SNI. One-way ANOVA. (F) Epidermal thickness was unchanged by 1-4 weeks of wheel running in uninjured mice, n=4-6. Student's t-Test. (G) Compared to uninjured controls, SNI, control hindpaw skin trended towards epidermal thinning that was unaffected by 2 or 12 hr/night of wheel running for 2 weeks, n=4. One-way ANOVA. In panels E and G, mean and SEM of 2 week, 2 hr/night uninjured, controls are represented by solid and dashed lines, respectively. Data are presented as mean \pm SEM. In all statistical analyses, a Bonferroni test was performed to correct for multiple comparisons.

denervation, center hindpaw total IENF density was quantified with β III tubulin after two weeks of exercise, 25 days post-SNI. IENF density of SNI animals was significantly reduced compared

to uninjured controls (One-way ANOVA: $p < 0.0001$) (Fig. 6E). However, wheel running for either 2 hr/night or 12 hr/night for 2 weeks did not improve IENF density of the ipsilateral hindpaw.

Intraepidermal fibers aid in the maintenance of the epidermis, and epidermal thinning is a consequence of denervation.^{6,33,53,68} Therefore, epidermal thickness was also quantified alongside IENF density. Epidermal thickness was measured as the vertical distance from the dermal-epidermal border to the outermost granular cell layer. In uninjured mice, 1 to 4 weeks of exercise training did not give rise to changes in epidermal thickness (Fig. 6F). In hindpaw skin of SNI, control mice, epidermal denervation was accompanied by epidermal thinning compared to uninjured controls, though the deficit did not reach statistical significance (Fig. 6G). Exercise for 2 weeks did not prevent SNI-induced epidermal thinning. This result was expected as recovery of epidermal thickness after nerve injury is thought to be the product of reinnervation^{19,53}, and exercise did not reduce SNI-induced denervation.

4.5 Discussion

We investigated the analgesic potential of voluntary wheel running in the contexts of acute inflammatory pain and neuropathic pain. We have shown that voluntary wheel running is not effective in attenuating formalin-induced nocifensive behavior or in improving SNI-induced mechanical hypersensitivity, muscle wasting, or skin denervation. We have also demonstrated that voluntary exercise training of uninjured animals does not cause changes in nociceptive thresholds, DRG excitability, or hindpaw innervation and epidermal thickness.

We report a lack of an effect of voluntary exercise training on nociceptive thresholds of uninjured animals. In contrast, Mathes *et al.* demonstrated significantly lower radiant heat tail flick response latencies, an end point we did not evaluate, following voluntary exercise in rats.⁵⁴ However, thermal and mechanical hindpaw withdrawal thresholds were unchanged in rats following forced swim training and in mice that had home cage running wheel access.^{64,66} Our findings, along with previous studies reporting no effect of voluntary exercise on nociceptive thresholds in uninjured rodents, are consistent with human studies that have demonstrated basal pain thresholds are usually unchanged in athletes compared to non-athletes.^{35,69} These data suggest that nociceptive thresholds are tightly regulated in an uninjured context to protect organisms from tissue damage without causing withdrawal from benign stimuli.

The finding that voluntary exercise training improves neither acute inflammatory pain responses nor recovery from peripheral nerve injury is in contrast to much of the existing literature. For instance, Kuphal *et al.* report that extended swimming for 9 days attenuates formalin-induced spontaneous pain.⁵⁰ Further, forced treadmill running has been shown to reverse thermal and mechanical hypersensitivity following both peripheral nerve and spinal cord injury.^{8,20,21,26,67}

A number of possible explanations may underlie our observation that exercise is not analgesic in the context of acute inflammatory and nerve injury-induced pain. Foremost, the

current study differs from much of the existing literature due to the use of voluntary as opposed to forced exercise, which is reportedly more stressful.^{16,46} For example, in a study conducted by Leasure and Jones, forced but not voluntary wheel running increased both anxiety-like behavior in the open field test and emotional defecation.⁵² Moving animals in and out of running wheel cages is a potential source of stress. However, in our study, both control and exercise mice were transferred to running wheel cages during exercise periods. The two groups therefore differed only in exercise training because control mice were provided with locked running wheels. As training progressed, we observed noticeably fewer fecal pellets at the end of each session, suggesting that mice were not chronically stressed when placed into their individual cages. Importantly, cages of exercised mice were visibly cleaner than that of control mice, implying that voluntary exercise may have exerted an anxiolytic effect in our paradigm, as reported by others.^{7,32,61} Collectively, our results indicate that the analgesic effect of exercise is potentially dependent on the nature of the exercise paradigm. Definitive proof that the different effects of voluntary versus forced exercise are related to stress will require additional investigation.

Another variable that could account for the differential effects of various exercise paradigms is exercise intensity. High intensity exercise has been shown to be more effective than low intensity exercise in reversing nerve injury-induced mechanical hypersensitivity in rats.⁶⁷ In our study, it is possible that voluntary wheel running is not of sufficient intensity to induce the physiological adaptations required to attenuate acute inflammatory pain or reverse SNI-induced mechanical hypersensitivity. However, voluntary wheel running has been shown to induce similar physiological adaptations as forced exercise paradigms that are effective in attenuating pain. Examples of these adaptations include increased expression of endogenous opioids and heat shock protein 72, as well as altered expression of growth factors.^{1,2,4,5,18,37,42,63,67} In addition, one study found that wheel running of comparable distance to

that observed in our study normalized behavioral hypersensitivity associated with pre-diabetic neuropathy.³⁷ These findings suggest that the exercise intensity of our paradigm is sufficient to engage the adaptive mechanisms contributing to exercise-induced analgesia. Other differences between our study and previous work such as social isolation and stress are more likely to contribute to the different outcomes.

Our study is the first to investigate the effects of exercise on SNI-induced neuropathic pain. While previous studies have shown that voluntary wheel running improves pain-like behavior in mouse models of chronic muscle pain and prediabetic neuropathy, voluntary wheel running failed to improve SNI-induced mechanical hypersensitivity in our hands.^{38,66} Our results suggest that voluntary exercise-induced analgesia may be specific to certain types of neuropathies. The absence of exercise-induced improvements in our study may be due to the severe – and perhaps less modifiable – nature of SNI, in which ligation of the common peroneal and tibial nerves results in marked denervation of the hindpaw. Context-specific benefits of exercise have also been observed clinically. For example, aerobic exercise improves quality of life only in some type II diabetes patients, who often suffer from diabetic peripheral neuropathy.^{27,41} It is also worth noting that clinically, improvements in quality of life do not necessitate improved pain ratings.⁵¹ Rodent pain behavioral assays such as von Frey rely on nociceptive withdrawal thresholds as opposed to an endpoint that more comprehensively reflects a global pain experience. It is possible that voluntary exercise training can improve quality of life of nerve-injured mice. Efforts have been made to develop assays that more appropriately reflect clinical pain, but developing non-reflexive pain measures for rodent nerve injury models has proven to be difficult.^{36,70}

Another distinguishing quality of SNI is that after injury von Frey testing occurs on the lateral aspect of the hindpaw, which is solely innervated by the spared sural nerve.²⁵ Thus, we tested the sensitivity of uninjured fibers – though perhaps in an injured environment. Other

rodent nerve injury models such as spinal nerve ligation and chronic constriction injury test hindpaw regions innervated by a combination of uninjured and injured afferents.^{11,24,55} Therefore, it is possible that exercise preferentially attenuates injury-induced dysfunction in injured nerve fibers.

The timing of exercise intervention relative to nerve injury may also contribute to the lack of exercise-induced improvements following SNI. Because we were interested in whether exercise can reverse nerve-injury induced hypersensitivity, we chose to initiate exercise training on POD 8-10, once SNI-induced mechanical hypersensitivity was verified across multiple post-injury time points. In contrast, prior studies initiated forced exercise training by POD 7.^{8,20,21,67} In these studies, exercise either attenuated the development of or improved existing mechanical hypersensitivity caused by peripheral nerve injury. Therefore, it is possible that our voluntary wheel running paradigm could improve SNI-induced mechanical hypersensitivity if training began closer to the time of injury, i.e. within one week. However, Stagg *et al.* tested if timing of exercise onset after spinal nerve ligation determines the time required to reverse mechanical hypersensitivity.⁶⁷ When exercise was initiated either 1 or 4 weeks after injury, mechanical hypersensitivity was reduced within 3 weeks. The time at which exercise intervention must be initiated in order to recover nociceptive thresholds remains an interesting question.

Our study evaluated the benefit of exercise in the prevention of acute formalin-induced pain and in the reversal of nerve injury-induced chronic pain. We have shown that our exercise paradigm does not prevent formalin-induced inflammatory pain. However, it is possible that our exercise paradigm would prevent the development of chronic pain induced by nerve injury. Inflammation-induced pain and nerve injury-induced pain share a number of common mechanisms; however, there are important differences.⁷² For instance, inflammatory pain is mediated through C fiber afferents, while neuropathic pain engages both C and A β fiber afferents.⁷² In addition, spontaneous pain is mediated by C fibers.²⁸ It is unknown which fiber

types are functionally influenced by voluntary wheel running in our study. Therefore, while our exercise paradigm did not prevent acute inflammation-induced spontaneous pain, it may prevent the development or delay the onset of SNI-induced mechanical hypersensitivity.

Lastly, it is also possible that exercise training of longer duration than our protocol of 2 weeks is needed to observe improvements of mechanical hypersensitivity following SNI. Duration of exercise training required to improve rodent pain-like symptoms varies in different studies. For instance, following chronic constriction injury of the mouse sciatic nerve, forced treadmill running within the first week of injury reduced mechanical hypersensitivity.²¹ If treadmill running persisted for more than one week, however, improvements in mechanical thresholds were not observed. Similarly, in rats that underwent sciatic nerve transection, 2 weeks of treadmill running was sufficient to return mechanical thresholds to baseline.⁴⁹ However, in other studies, the effects of exercise training are not apparent unless exercise persists for periods longer than 2 weeks after injury. Reductions in mechanical and heat hypersensitivity caused by spinal nerve ligation in rats did not develop until after the third week of treadmill running.⁶⁷ Further, Groover *et al.* first observed reversal of prediabetic neuropathy-induced mechanical and visceral hypersensitivity after 8 weeks of voluntary wheel running.³⁸

The injury models examined in our study largely represent cutaneous pain. Even so, we cannot exclude injury to deep tissue or effects on deep tissue afferents (muscle) in either model. The SNI surgical procedure causes minimal damage to muscle; likewise, subcutaneous formalin injections could injure hindpaw muscle. However, the spontaneous nocifensive behavior resulting from subcutaneous formalin results from activation of cutaneous afferents and hindpaw von Frey is a measure of SNI-induced cutaneous hypersensitivity.^{17,59} Our study therefore evaluates the effect of voluntary exercise on cutaneous pain. Measures of deep tissue pain in SNI mice after exercise training, though not within the scope of our study, would address

whether voluntary exercise can reverse nerve injury-induced deep tissue pain, even though cutaneous hypersensitivity remains unchanged.

To conclude, the current study demonstrates that voluntary exercise training of uninjured animals does not alter nociceptive thresholds, sensory neuron excitability, or hindpaw intraepidermal innervation. We also demonstrate that voluntary wheel running fails to attenuate formalin-induced acute inflammatory pain and SNI-induced mechanical hypersensitivity, muscle wasting, and hindpaw denervation. When compared to existing literature, these results suggest that voluntary and forced exercise paradigms may have different analgesic potential. Further, the analgesic efficacy of voluntary exercise may be influenced by a number of variables including type of injury, timing of intervention, duration of exercise, and exercise intensity.

4.6 Acknowledgements

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4.7 Author Contributions

Designed the experiments: TDS, BAC, JPG, RWG. Performed the experiments: TDS, BAC, JPG. Analyzed the data: TDS. Wrote the paper: TDS. All authors reviewed, edited, and approved the manuscript.

4.8 References

1. Almeida C, Demaman A, Kusuda R, Cadetti F, Ida M, Sousa TA, Zanon S, Silveira LR, Lucas G: Exercise therapy normalizes BDNF upregulation and glial hyperactivity in a mouse model of neuropathic pain. *Pain* 156:504–13, 2015.
2. Armada-da-Silva P a S, Pereira C, Amado S, Veloso AP: Role of physical exercise for improving posttraumatic nerve regeneration. *Int Rev Neurobiol* 109:125–49, 2013.
3. Baldo G, Wozniak DF, Ohlemiller KK, Zhang Y, Giugliani R, Ponder KP: Retroviral-vector-mediated gene therapy to mucopolysaccharidosis I mice improves sensorimotor impairments and other behavioral deficits. *J Inherit Metab Dis* 36:499–512, 2013.
4. Belter JG, Carey H V, Garland T: Effects of voluntary exercise and genetic selection for high activity levels on HSP72 expression in house mice. *J Appl Physiol* 96:1270–6, 2004.
5. Bement MKH, Sluka KA: Low-intensity exercise reverses chronic muscle pain in the rat in a naloxone-dependent manner. *Arch Phys Med Rehabil* 86:1736–40, 2005.
6. Benrath J, Zimmermann M, Gillardon F: Substance P and nitric oxide mediate wound healing of ultraviolet photodamaged rat skin: evidence for an effect of nitric oxide on keratinocyte proliferation. *Neurosci Lett* 200:17–20, 1995.
7. Binder E, Droste SK, Ohi F, Reul JMH: Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice. *Behav Brain Res* 155:197–206, 2004.
8. Bobinski F, Martins DF, Bratti T, Mazzardo-Martins L, Winkelmann-Duarte EC, Guglielmo LG a, Santos a RS: Neuroprotective and neuroregenerative effects of low-intensity aerobic exercise on sciatic nerve crush injury in mice. *Neuroscience* 194:337–48, 2011.
9. Boeltz T, Ireland M, Mathis K, Nicolini J, Poplavski K, Rose SJ, Wilson E, English AW: Effects of treadmill training on functional recovery following peripheral nerve injury in rats. *J Neurophysiol* 109:2645–57, 2013.
10. Bonaldo P, Sandri M: Cellular and molecular mechanisms of muscle atrophy. *Dis Model Mech* 6:25–39, 2013.
11. Boucher TJ, Okuse K, Bennett DLH, Munson JB, Wood JN, McMahon SB: Potent Analgesic Effects of GDNF in Neuropathic Pain States. *Science* (80-) 290:124–7, 2000.
12. Brenner DS, Golden JP, Gereau RW: A novel behavioral assay for measuring cold sensation in mice. *PLoS One* 7:e39765, 2012.
13. Brenner DS, Golden JP, Vogt SK, Gereau RW: A simple and inexpensive method for determining cold sensitivity and adaptation in mice. *J Vis Exp* :e52640, 2015.
14. Butler RK, Finn DP: Stress-induced analgesia. *Prog Neurobiol* 88:184–202, 2009.
15. Caggiula a. R, Perkins K a., Saylor S, Epstein LH: Different methods of assessing nicotine-induced antinociception may engage different neural mechanisms. *Psychopharmacology (Berl)* 122:301–6, 1995.
16. Carmody J, Cooper K: Swim stress reduces chronic pain in mice through an opioid

- mechanism. *Neurosci Lett* 74:358–63, 1987.
17. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53:55–63, 1994.
 18. Chen Y-W, Hsieh P-L, Chen Y-C, Hung C-H, Cheng J-T: Physical exercise induces excess hsp72 expression and delays the development of hyperalgesia and allodynia in painful diabetic neuropathy rats. *Anesth Analg* 116:482–90, 2013.
 19. Chiang HY, Huang IT, Chen WP, Chien HF, Shun CT, Chang YC, Hsieh ST: Regional difference in epidermal thinning after skin denervation. *Exp Neurol* 154:137–45, 1998.
 20. Cobiañchi S, Casals-Diaz L, Jaramillo J, Navarro X: Differential effects of activity dependent treatments on axonal regeneration and neuropathic pain after peripheral nerve injury. *Exp Neurol* 240:157–67, 2013.
 21. Cobiañchi S, Marinelli S, Florenzano F, Pavone F, Luvisetto S: Short- but not long-lasting treadmill running reduces allodynia and improves functional recovery after peripheral nerve injury. *Neuroscience* 168:273–87, 2010.
 22. Courneya KS, McKenzie DC, Mackey JR, Gelmon K, Friedenreich CM, Yasui Y, Reid RD, Cook D, Jespersen D, Proulx C, Dolan LB, Forbes CC, Wooding E, Trinh L, Segal RJ: Effects of exercise dose and type during breast cancer chemotherapy: multicenter randomized trial. *J Natl Cancer Inst* 105:1821–32, 2013.
 23. Daenen L, Varkey E, Kellmann M, Nijs J: Exercise, not to exercise, or how to exercise in patients with chronic pain? Applying science to practice. *Clin J Pain* 31:108–14, 2015.
 24. Decosterd I, Ji RR, Abdi S, Tate S, Woolf CJ: The pattern of expression of the voltage-gated sodium channels Nav1.8 and Nav1.9 does not change in uninjured primary sensory neurons in experimental neuropathic pain models. *Pain* 96:269–77, 2002.
 25. Decosterd I, Woolf CJ: Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87:149–58, 2000.
 26. Detloff MR, Smith EJ, Molina DQ, Ganzer PD, Houlié JD: Acute exercise prevents the development of neuropathic pain and the sprouting of non-peptidergic (GDNF- and artemin-responsive) c-fibers after spinal cord injury. *Exp Neurol* 255:38–48, 2014.
 27. Dixit S, Maiya A, Shastry B: Effect of aerobic exercise on quality of life in population with diabetic peripheral neuropathy in type 2 diabetes: a single blind, randomized controlled trial. *Qual Life Res* 23:1629–40, 2013.
 28. Djouhri L, Koutsikou S, Fang X, McMullan S, Lawson SN: Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. *J Neurosci* 26:1281–92, 2006.
 29. Dobson JL, McMillan J, Li L: Benefits of exercise intervention in reducing neuropathic pain. *Front Cell Neurosci* 8:1–9, 2014.
 30. Draxler P, Honsek SD, Forsthuber L, Hadschieff V, Sandkühler J: VGlut3+ Primary Afferents Play Distinct Roles in Mechanical and Cold Hypersensitivity Depending on Pain Etiology. *J Neurosci* 34:12015–28, 2014.

31. Dubreucq S, Marsicano G, Chaouloff F: Emotional consequences of wheel running in mice: Which is the appropriate control? *Hippocampus* 21:239–42, 2011.
32. Duman CH, Schlesinger L, Russell DS, Duman RS: Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. *Brain Res* 1199:148–58, 2008.
33. Duraku LS, Hossaini M, Hoendervangers S, Falke LL, Kambiz S, Mudera VC, Holstege JC, Walbeehm ET, Ruigrok TJH: Spatiotemporal dynamics of re-innervation and hyperinnervation patterns by uninjured CGRP fibers in the rat foot sole epidermis after nerve injury. *Mol Pain* 8:61, 2012.
34. Gereau RW, Sluka KA, Maixner W, Savage SR, Price TJ, Murinson BB, Sullivan MD, Fillingim RB: A pain research agenda for the 21st century. *J Pain* 15:1203–14, 2014.
35. Geva N, Defrin R: Enhanced pain modulation among triathletes: a possible explanation for their exceptional capabilities. *Pain* 154:2317–23, 2013.
36. Grace PM, Strand KA, Maier SF, Watkins LR: Suppression of voluntary wheel running in rats is dependent on the site of inflammation: evidence for voluntary running as a measure of hind paw-evoked pain. *J Pain* 15:121–8, 2014.
37. Groover AL, Ryals JM, Guilford BL, Wilson NM, Christianson J a, Wright DE: Exercise-Mediated Improvements in Painful Neuropathy Associated with Pre-Diabetes in Mice. *Pain* , 2013.
38. Groover AL, Ryals JM, Guilford BL, Wilson NM, Christianson JA, Wright DE: Exercise-mediated improvements in painful neuropathy associated with prediabetes in mice. *Pain* 154:2658–67, 2013.
39. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception. *Pain* 32:77–88, 1988.
40. Hawkley LC, Cole SW, Capitanio JP, Norman GJ, Cacioppo JT: Effects of social isolation on glucocorticoid regulation in social mammals. *Horm Behav* 62:314–23, 2012.
41. van der Heijden MMP, van Dooren FEP, Pop VJM, Pouwer F: Effects of exercise training on quality of life, symptoms of depression, symptoms of anxiety and emotional well-being in type 2 diabetes mellitus: a systematic review. *Diabetologia* 56:1210–25, 2013.
42. Hoffmann P, Terenius L, Thorén P: Cerebrospinal fluid immunoreactive β -endorphin concentration is increased by voluntary exercise in the spontaneously hypertensive rat. *Regul Pept* 28:233–9, 1990.
43. Hurkmans E, van der Giesen F, Vliet Vlieland T, Schoones J, Van den Ende E: Dynamic exercise programs (aerobic capacity and / or muscle strength training) in patients with rheumatoid arthritis (Review). *Cochrane Libr* :4–6, 2009.
44. Ilha J, Araujo RT, Malysz T, Hermel EES, Rigon P, Xavier LL, Achaval M: Endurance and resistance exercise training programs elicit specific effects on sciatic nerve regeneration after experimental traumatic lesion in rats. *Neurorehabil Neural Repair* 22:355–66, 2008.
45. Institute of Medicine: Relieving Pain in America : A Blueprint for Transforming Prevention, Care, Education, and Research. 2011.

46. Ke Z, Yip SP, Li L, Zheng XX, Tong KY: The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: A rat brain ischemia model. *PLoS One* 6:e16643, 2011.
47. Klusáková I, Dubový P: Experimental models of peripheral neuropathic pain based on traumatic nerve injuries - an anatomical perspective. *Ann Anat* 191:248–59, 2009.
48. Koltyn KF: Analgesia Following Exercise: A Review. *Sports Med* 29:85–98, 2000.
49. Korb A, Bonetti LV, da Silva SA, Marcuzzo S, Ilha J, Bertagnolli M, Partata WA, Faccioni-Heuser MC: Effect of treadmill exercise on serotonin immunoreactivity in medullary raphe nuclei and spinal cord following sciatic nerve transection in rats. *Neurochem Res* 35:380–9, 2010.
50. Kuphal KE, Fibuch EE, Taylor BK: Extended swimming exercise reduces inflammatory and peripheral neuropathic pain in rodents. *J Pain* 8:989–97, 2007.
51. Lamé IE, Peters ML, Vlaeyen JWS, Kleef M V., Patijn J: Quality of life in chronic pain is more associated with beliefs about pain, than with pain intensity. *Eur J Pain* 9:15–24, 2005.
52. Leasure JL, Jones M: Forced and voluntary exercise differentially affect brain and behavior. *Neuroscience* 156:456–65, 2008.
53. Li Y, Hsieh ST, Chien HF, Zhang X, McArthur JC, Griffin JW: Sensory and motor denervation influence epidermal thickness in rat foot glabrous skin. *Exp Neurol* 147:452–62, 1997.
54. Mathes WF, Kanarek RB: Chronic running wheel activity attenuates the antinociceptive actions of morphine and morphine-6-glucuronide administration into the periaqueductal gray in rats. *Pharmacol Biochem Behav* 83:578–84, 2006.
55. Michaelis M, Liu X, Jänig W: Axotomized and intact muscle afferents but no skin afferents develop ongoing discharges of dorsal root ganglion origin after peripheral nerve lesion. *J Neurosci* 20:2742–8, 2000.
56. Moraska a, Deak T, Spencer RL, Roth D, Fleshner M: Treadmill running produces both positive and negative physiological adaptations in Sprague-Dawley rats. *Am J Physiol Regul Integr Comp Physiol* 279:R1321–9, 2000.
57. Noble EG, Moraska a, Mazzeo RS, Roth D a, Olsson MC, Moore RL, Fleshner M: Differential expression of stress proteins in rat myocardium after free wheel or treadmill run training. *J Appl Physiol* 86:1696–701, 1999.
58. Ploughman M, Granter-Button S, Chernenko G, Attwood Z, Tucker B a., Mearow KM, Corbett D: Exercise intensity influences the temporal profile of growth factors involved in neuronal plasticity following focal ischemia. *Brain Res* 1150:207–16, 2007.
59. Ren K, Dubner R: Inflammatory Models of Pain and Hyperalgesia. *ILAR J* 40:111–8, 1999.
60. Rubinstein M, Mogili JS, Japntll M, Chant EC, Allentt RG, Lowt MJ: Absence of opioid stress-induced analgesia in mice lacking f3-endorphin by site-directed mutagenesis. *Proc*

Natl Acad Sci 93:3995–4000, 1996.

61. Salam JN, Fox JH, Detroy EM, Guignon MH, Wohl DF, Falls WA: Voluntary exercise in C57 mice is anxiolytic across several measures of anxiety. *Behav Brain Res* 197:31–40, 2009.
62. Sciolino NR, Holmes P V: Exercise offers anxiolytic potential: a role for stress and brain noradrenergic-galaninergic mechanisms. *Neurosci Biobehav Rev* 36:1965–84, 2012.
63. Sharma NK, Ryals JM, Gajewski BJ, Wright DE: Aerobic exercise alters analgesia and neurotrophin-3 synthesis in an animal model of chronic widespread pain. *Phys Ther* 90:714–25, 2010.
64. Shen J, Fox LE, Cheng J, Clinic C: Swim Therapy Reduces Mechanical Allodynia and Thermal Hyperalgesia Induced by Chronic Constriction Nerve Injury in Rats. *Pain Med* 14:516–25, 2013.
65. Shyu B-C, Andersson SA, Thorén P: Endorphin mediated increase in pain threshold induced by long-lasting exercise in rats. *Life Sci* 30:833–40, 1982.
66. Sluka KA, O'Donnell JM, Danielson J, Rasmussen LA: Regular physical activity prevents development of chronic pain and activation of central neurons. *J Appl Physiol* 114:725–33, 2013.
67. Stagg NJ, Mata HP, Ibrahim M, Henriksen EJ, Ph D, Porreca F, Vanderah TW, Malan TP: Regular Exercise Reverses Sensory Hypersensitivity in a Rat Neuropathic Pain Model. *Pain Med* 114:940–8, 2011.
68. Takahasi K, Nakanishi S, Imamura S: Direct Effects of Cutaneous Neuropeptides on Adenylyl Cyclase Activity and Proliferation in a Keratinocyte Cell Line: Stimulation fo Cyclic AMP Formation by CGRP and VIP/PHM, and Inhibition by NPY Through G Protein - Coupled Receptors. *J Invest Dermatol* 101:646–51, 1993.
69. Tesarz J, Schuster AK, Hartmann M, Gerhardt A, Eich W: Pain perception in athletes compared to normally active controls: a systematic review with meta-analysis. *Pain* 153:1253–62, 2012.
70. Urban R, Scherrer G, Goulding EH, Tecott LH, Basbaum AI: Behavioral indices of ongoing pain are largely unchanged with tissue or nerve-injury induced mechanical hypersensitivity. *Pain* 152:990–1000, 2011.
71. Wright A, Ph D, Sluka KA: Nonpharmacological Treatments for Musculoskeletal Pain. *Clin J Pain* 17:33–46, 2001.
72. Xu Q, Yaksh TL: A brief comparison of the pathophysiology of inflammatory versus neuropathic pain. *Curr Opin Anaesthesiol* 24:400–7, 2011.
73. Zhao L, Lv G, Jiang S, Yan Z, Sun J, Wang L, Jiang D: Morphological differences in skeletal muscle atrophy of rats with motor nerve and/or sensory nerve injury. *Neural Regen Res* 7:2507–15, 2012.

Chapter 5

Conclusions and Future Directions

Over the past century, we have gained a remarkable understanding of the mechanisms underlying nociception, acute pain, and chronic pain. Unfortunately, this knowledge has not led to sufficient interventions to ease the suffering of the millions of people with chronic pain conditions. This dissertation describes and evaluates multiple approaches that could be used by basic scientists to test the translational potential of basic pain research findings. One approach includes the development and implementation of rodent behavioral assays that reflect the somatosensory as well as the cognitive and affective components characteristic of human pain (Chapter 2). Another approach entails utilizing human sensory neurons to assess whether mechanisms of peripheral sensitization blockade translate from rodents to human (Chapter 3). Lastly, we assess whether exercise-induced analgesia, a nonpharmacological intervention for pain relief, can be modeled in mice (Chapter 4). While these approaches for bridging the translational gap between rodent and human pain research were met with varied success, they raise important considerations for pain researchers.

5.1 Can we use rodents to model human pain?

In Chapter 2, we evaluated whether voluntary behaviors are interrupted in common rodent pain models. While chronic pain impairs physical and social activities and increases anxiety symptoms in humans^{8,11,13,25,32,37}, we found that inflammation and nerve injury minimally interfere, if at all, with physical/social activity or anxiety-like behavior in mice. Overwhelmingly negative findings from our group and others^{22,35} have led some pain researchers to question whether we can or should use rodents to model human chronic pain and assess putative analgesics.^{2,21,36} Rodent models frequently fail to recapitulate the entirety of human disease states⁹, and it is perhaps unreasonable to expect so given different evolutionary pressures faced by rodents and humans. However, we support the continued use of rodents for preclinical pain research. Existing rodent models and pain-related behavioral assays of hypersensitivity

continue to provide valuable insights regarding the somatosensory aspects of chronic pain.^{16,38} Therefore, the use of traditional measures of hypersensitivity in combination with newly developed pain-related measures of cognitive and motivational aspects of pain will provide an enhanced understanding of the translational potential of preclinical findings.

5.2 Are innate rodent behaviors interrupted in pain models?

We assessed whether rodent models of persistent pain produce changes in voluntary behavior analogous to human behavioral changes seen in chronic pain conditions. However, an alternative approach in the development of non-reflexive measure of pain-like behavior is to determine whether innate rodent behaviors are altered following injury. For instance, impaired nest building, burrowing, and escape from bright light have all been suggested as pain-related suppressed behaviors in rodents and are broadly interpreted as indicators of reduced general well being.^{1,6,12,23,24} Importantly, these endpoints demonstrate predictive validity. Known analgesics such as NSAIDs or morphine can reverse inflammation- or nerve injury-induced suppression of nest building, burrowing, and bright light avoidance.^{1,12,24} In contrast, drugs that fail to provide analgesia clinically, such as kappa opioid receptor antagonists, do not.^{12,24} Further investigation is required to assess the utility of suppressed innate behaviors in preclinical pain research. For instance, the duration of inflammation-induced reductions in nest building appears equally short-lived (3-4 days) as the reductions we report in voluntary wheel running and gait in Chapter 2.²⁴ In this regard, measures of suppressed innate rodent behavior may possess some of the same limitations as voluntary behavioral measures of pain.

Nevertheless, pain-related suppressed behaviors can provide information that otherwise goes undetected in tests of hypersensitivity. Suppression of innate behaviors does not correlate with mechanical hypersensitivity, and thus may reflect either spontaneous or global pain, rather than somatosensory thresholds alone.^{1,23} Further, measures of pain-suppressed behaviors

possess greater predictive validity than reflex withdrawal assays when assessing the efficacy of putative analgesics.¹ In reflex/withdrawal tests, an increase in paw withdrawal threshold or latency is interpreted as a reduction in pain-like behavior. However, this could also reflect sedation or motor side effects caused by a drug. In contrast, the reversal of pain-related suppressed behaviors requires enhanced animal activity. Thus, endpoints such as burrowing decrease the likelihood of obtaining false-positive analgesic effects from pharmacological interventions. Taken together, measures of innate rodent pain-related suppressed behaviors represent an ethological approach to evaluating the somatosensory and affective components of pain in rodents.

5.3 What terminology should be used to describe rodent pain-related behaviors?

The recent efforts to develop voluntary, non-reflexive endpoints prompts pain researchers to evaluate the terminology used to describe rodent pain-like behavior. At present, it remains unclear whether behavioral changes observed after injury in rodents reflect pain, which is defined as a complex sensory, emotional, and cognitive experience. Thus, using the phrase “pain behavior” synonymously with mechanical and/or thermal hypersensitivity extrapolates behavioral observations and in turn anthropomorphizes rodents. This commonly used simplified terminology can be advantageous in that it increases the accessibility of pain research findings to those in other fields and in turn aids cross-sectional research, such as the interplay between drug addiction and chronic pain. Further, it is difficult to completely avoid anthropomorphizing terminology when discussing *in vitro* and animal models of clinical phenomena, such as peripheral analgesia in Chapter 3 and exercise-induced analgesia in Chapter 4.

The use of simplified terminology is problematic when striving to identify behavioral changes triggered by damage to the somatosensory sensory system, and a more precise

terminology for endpoints is therefore needed. This new terminology certainly requires consensus among pain researchers. We propose that the term “sensitization”, in reference to the mechanisms of peripheral and central sensitization thought to manifest in behavioral changes in humans and rodents, may be more appropriate than “pain”. Thus, throughout Chapters 2 and 4, we used the term “hypersensitivity” rather than “allodynia” or “hyperalgesia” to avoid anthropomorphizing the reduction in mouse mechanical and/or thermal paw withdrawal thresholds. Minor adjustments such as this in terminology used to describe behavioral changes associated with rodent injury models can offer substantial clarity as we continue to expand our repertoire of rodent pain-related assays.

5.4 Which molecular mechanisms mediate peripheral analgesia in humans?

In Chapter 3 we demonstrated that mGlu2/3 receptor activation suppresses inflammation-induced TRPV1 sensitization in mouse sensory neurons. However, this mechanism of sensory neuron sensitization blockade did not translate in human sensory neurons. Which molecular mechanisms underlie this species difference in mGluR2/3 function is a compelling question. However, human tissue is a limited and valuable resource. We should therefore prioritize the use of human sensory neurons in studies that seek to understand human sensory neuron physiology rather than species differences.

We previously showed that mGlu2/3 receptor activation blocks PGE₂-induced membrane hyperexcitability in human sensory neurons.⁵ This observation establishes mGluR2/3 as putative peripheral analgesic targets in human. However, the underlying intracellular signaling pathway(s) engaged by mGluR2/3 that reduce hyperexcitability in human sensory neurons require further investigation. One potential mechanism includes enhanced activity of G protein-coupled inward rectifying potassium channels (GIRKs). GIRKs, stimulated via Gβγ subunits of Gi proteins, are the major type of potassium channels activated by GPCRs.¹⁹ GIRK functional

expression has been demonstrated in rat sensory neurons and has been proposed to be required for peripheral opioid-induced analgesia by decreasing neuronal excitability.^{19,20,26} Notably, GIRK mRNA and protein has also been detected in human sensory neurons.^{26,28} Like opioid receptors, group II mGluRs have been shown to functionally couple with GIRK channels in heterologous systems as well as in rodent and avian neurons.^{7,15,29,31} It would therefore be interesting to test whether the same functional coupling occurs in human sensory neurons and mediates mGluR2/3-induced suppression of membrane hyperexcitability. This could be achieved by determining whether inflammation-induced membrane hyperexcitability is occluded when a group II mGluR agonist is coapplied with a GIRK inhibitor. Experiments that aim to elucidate the intracellular signaling pathways activated in human sensory neurons by putative analgesics such as mGluR2/3 agonists can reveal unexpected species differences in sensory neuron physiology. Thus, using human sensory neurons to validate preclinical findings will improve our understanding of human physiology and inform the design of future clinical trials for pain relief.

5.5 Does voluntary exercise reduce pain-like behavior in rodents?

In Chapter 4, we evaluated whether voluntary wheel running could be used as a rodent model of exercise-induced analgesia with the intent of investigating the molecular mechanisms that mediate this clinically observed phenomena. Interestingly, we found that voluntary exercise did not alter stimulus-evoked paw withdrawal thresholds and/or nocifensive behaviors under basal conditions or in common mouse models of inflammation and nerve injury. At the time of publication, this was one of the first studies that utilized a voluntary rather than a forced exercise paradigm to investigate exercise-induced analgesia in rodents. Given that forced, but not voluntary, exercise increases activation of the hypothalamic-pituitary-adrenal axis^{14,33}, our null findings prompt the question: If, and to what extent, do results of previous studies that utilized

forced exercise paradigms reflect a combination of exercise- and stress-induced analgesia? To answer this question, a side-by-side comparison evaluating the effects of forced versus voluntary exercise on rodent injury-induced pain-like behavior is required.

While such a comparative study has not yet been completed, since the publication of our findings, multiple studies have demonstrated voluntary exercise-induced analgesia in rodents. For instance, voluntary wheel running *prevents* the development of hypersensitivity and/or weight bearing asymmetry induced by nerve injury, intra-articular inflammation, as well as chronic muscle pain.^{4,10,18} These recent observations contradict our findings; however, this contradiction may result from different experimental conditions between studies (discussed below). The benefits of exercise in nerve injury and chronic muscle pain models have been attributed to suppression of neuroinflammation at the site of injury and spinal cord in the form of either 1) decreased release of inflammatory cytokines¹⁰ or 2) a phenotypic switching of macrophages that enhances the release of anti-inflammatory cytokines.¹⁸ Interestingly, attenuation of nerve injury-induced mechanical hypersensitivity by forced treadmill running has been associated with decreased activation of spinal microglia that release proinflammatory cytokines.^{3,34} Thus, it is possible that voluntary and forced exercise engage the same molecular mechanisms to reduce pain-like behavior in rodents. Taken together, recent findings suggest that prior voluntary exercise is protective against the development of persistent pain-like behavior in rodents. This observation appears to extend to humans, as there is a lower prevalence of chronic pain in regularly exercising patient populations.¹⁷ For this reason, it has been suggested that chronic pain may represent a sedentary lifestyle-related disease.³⁰

Preventing the onset of chronic pain is an ideality. Instead, clinicians need interventions to treat established chronic pain conditions. Therefore, it is most relevant to assess whether exercise *reverses* pain-like behavior in rodent pain models. Unfortunately, little progress has been made in this regard. Since our publication, only two studies have evaluated voluntary

wheel running following nerve injury or intra-articular inflammation in rats.^{10,27} Interestingly, in contrast to our null findings, both studies show reversal of injury-induced hypersensitivity within 2 weeks of wheel access, regardless of the onset of exercise. This discrepancy may be explained by a variety of differences in study design such as extent of wheel access (restricted versus homecage), injury model, subject species, etc. Thus, many questions remain regarding the analgesic potential of voluntary exercise in rodent models. Future investigations are also needed to determine whether this phenomenon is replicable and/or extends to additional pain models (i.e. visceral pain), as well as to gain insights regarding the molecular mechanisms that mediate voluntary exercise-induced analgesia after injury. Addressing these knowledge gaps may provide valuable insights regarding which clinical chronic pain conditions would be most improved by exercise interventions.

5.6 References

1. Andrews N, Legg E, Lisak D, Issop Y, Richardson D, Harper S, Huang W, Burgess G, Machin I, Rice ASC: Spontaneous burrowing behaviour in the rat is reduced by peripheral nerve injury or inflammation associated pain. *Eur J Pain* 16:485–95, 2012.
2. Le Bars D, Gozariu M, Cadden SW: Animal models of nociception. *Pharmacol Rev* 53:597–652, 2001.
3. Cobianchi S, Marinelli S, Florenzano F, Pavone F, Luvisetto S: Short- but not long-lasting treadmill running reduces allodynia and improves functional recovery after peripheral nerve injury. *Neuroscience* 168:273–87, 2010.
4. Cormier J, Cone K, Lanpher J, Kinens A, Henderson T, Liaw L, Bilsky EJ, King T, Rosen CJ, Stevenson GW: Exercise reverses pain-related weight asymmetry and differentially modulates trabecular bone microarchitecture in a rat model of osteoarthritis. *Life Sci* 180:51–9, 2017.
5. Davidson S, Golden JP, Copits B a, Ray PR, Vogt SK, Price TJ, Schmidt RE, Ghetti A, Gereau IV RW: Group 2 mGluRs suppress hyperexcitability in mouse and human nociceptors. *Pain* 157:2081–8, 2016.
6. Deacon RMJ: Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. *Nat Protoc* 1:118–21, 2006.
7. Dutar P, Vu HM, Perkel DJ: Pharmacological characterization of an unusual mGluR-evoked neuronal hyperpolarization mediated by activation of GIRK channels. *Neuropharmacology* 38:467–75, 1999.
8. Dysvik E, Lindstrøm TC, Eikeland OJ, Natvig GK: Health-related quality of life and pain beliefs among people suffering from chronic pain. *Pain Manag Nurs* 5:66–74, 2004.
9. Elsea SH, Lucas RE: The Mousetrap: What We Can Learn When the Mouse Model Does Not Mimic the Human Disease. *ILAR J* 43:66–79, 2002.
10. Grace PM, Fabisiak TJ, Green-Fulham SM, Anderson ND: Prior voluntary wheel running attenuates neuropathic pain. *Pain* 157:2012–23, 2016.
11. Gureje O: Comorbidity of pain and anxiety disorders. *Curr Psychiatry Rep* 10:318–22, 2008.
12. Harte SE, Meyers JB, Donahue RR, Taylor BK, Morrow TJ: Mechanical Conflict System: A Novel Operant Method for the Assessment of Nociceptive Behavior. *PLoS One* 11:e0150164, 2016.
13. He D, Grant B, Holden RR, Gilron I: Methodology for self-report of rest pain (or spontaneous pain) vs evoked pain in chronic neuropathic conditions. *PAIN Reports* 2:e587, 2017.
14. Ke Z, Yip SP, Li L, Zheng XX, Tong KY: The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: A rat brain ischemia model. *PLoS One* 6:e16643, 2011.

15. Knoflach F, Kemp JA: Metabotropic glutamate group II receptors activate a G protein-coupled inwardly rectifying K⁺ current in neurones of the rat cerebellum. *J Physiol* 509 (Pt 2):347–54, 1998.
16. Kontinen VK, Meert TF: Predictive validity of neuropathic pain models in pharmacological studies with a behavioural outcome in the rat: a systematic review. In: Dostrovsky JO, Carr DB, Koltzenburg M, editors. *Proceedings of the 10th World Congress on Pain* page 489–982002.
17. Landmark T, Romundstad PR, Borchgrevink PC, Kaasa S, Dale O: Longitudinal Associations between Exercise and Pain in the General Population - The HUNT Pain Study. *PLoS One* 8:e65279, 2013.
18. Leung A, Gregory NS, Allen L-AH, Sluka KA: Regular physical activity prevents chronic pain by altering resident muscle macrophage phenotype and increasing interleukin-10 in mice. *Pain* 157:70–9, 2016.
19. Lüscher C, Slesinger PA: Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nat Rev Neurosci* 11:301–15, 2010.
20. Lyu C, Mulder J, Barde S, Sahlholm K, Zeberg H, Nilsson J, Århem P, Hökfelt T, Fried K, Shi T-JS: G Protein-Gated Inwardly Rectifying Potassium Channel Subunits 1 and 2 are Down-Regulated in Rat Dorsal Root Ganglion Neurons and Spinal Cord after Peripheral Axotomy. *Mol Pain* 11:1–19, 2015.
21. Mogil JS: Animal models of pain: progress and challenges. *Nat Rev Neurosci* 10:283–94, 2009.
22. Mogil JS, Graham AC, Ritchie J, Hughes SF, Austin J-S, Schorscher-Petcu A, Langford DJ, Bennett GJ: Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. *Mol Pain* 6:34, 2010.
23. Muralidharan A, Kuo A, Jacob M, Lourdesamy JS, Melo Sores Pinho De Carvalho L, Nicholson JR, Corradini L, Smith MT: Comparison of burrowing and stimuli-evoked pain behaviors as end-points in rat models of inflammatory pain and peripheral neuropathic pain. *Front Behav Neurosci* 10:88, 2016.
24. Negus SS, Neddenriep B, Altarifi AA, Carroll FI, Leiti MD, Miller LL: Effects of ketoprofen, morphine, and kappa opioids on pain-related depression of nesting in mice. *Pain* 156:1153–60, 2015.
25. Nicholson B, Verma S: Comorbidities in chronic neuropathic pain. *Pain Med* 5:S9–27, 2004.
26. Nockemann D, Rouault M, Labuz D, Hublitz P, Mcknelly K, Reis FC, Stein C, Heppenstall PA: The K⁺ channel GIRK2 is both necessary and sufficient for peripheral opioid-mediated analgesia. *EMBO Mol Med* 5:1263–77, 2013.
27. Pitcher MH, Tarum F, Rauf IZ, Low LA, Bushnell C: Modest Amounts of Voluntary Exercise Reduce Pain- and Stress-Related Outcomes in a Rat Model of Persistent Hind Limb Inflammation. *J Pain* 18:687–701, 2017.

28. Price TJ, Dussor G: DRG TXome Database [Internet]. [cited 2017 Sep 12]. Available from: <https://www.utdallas.edu/bbs/painneurosciencelab/DRGtranscriptome/search.php>
29. Saugstad JA, Segerson TP, Westbrook GL: Metabotropic glutamate receptors activate G-protein-coupled inwardly rectifying potassium channels in *Xenopus* oocytes. *J Neurosci* 16:5979–85, 1996.
30. Senba E, Kami K: A new aspect of chronic pain as a lifestyle-related disease. *Neurobiol Pain* 1:6–15, 2017.
31. Sharon D, Vorobiov D, Dascal N: Positive and Negative Coupling of the Metabotropic Glutamate Receptors to a G Protein–activated K⁺ Channel, GIRK, in *Xenopus* Oocytes. *J Gen Physiol* 109:477–90, 1997.
32. Smith BH, Torrance N: Epidemiology of neuropathic pain and its impact on quality of life. *Curr Pain Headache Rep* 16:191–8, 2012.
33. Svensson M, Rosvall P, Boza-Serrano A, Andersson E, Lexell J, Deierborg T: Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia. *Neurobiol Stress* 5:8–18, 2016.
34. Tsuda M, Inoue K, Salter MW: Neuropathic pain and spinal microglia: A big problem from molecules in “small” glia. *Trends Neurosci* 28:101–7, 2005.
35. Urban R, Scherrer G, Goulding EH, Tecott LH, Basbaum AI: Behavioral indices of ongoing pain are largely unchanged with tissue or nerve-injury induced mechanical hypersensitivity. *Pain* 152:990–1000, 2011.
36. Vierck CJ, Hansson PT, Yeziarski RP: Clinical and pre-clinical pain assessment: are we measuring the same thing? *Pain* 135:7–10, 2008.
37. Wallace LS, Wexler RK, McDouggle L, Miser WF, Haddox JD: Voices that may not otherwise be heard: A qualitative exploration into the perspectives of primary care patients living with chronic pain. *J Pain Res* 7:291–9, 2014.
38. Whiteside GT, Adedoyin A, Leventhal L: Predictive validity of animal pain models? A comparison of the pharmacokinetic-pharmacodynamic relationship for pain drugs in rats and humans. *Neuropharmacology* 54:767–75, 2008.

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EDUCATION & TRAINING

Washington University in St. Louis, St. Louis, MO (08/2012-Present)

Ph.D., Neurosciences (expected 12/2017)

Dissertation: Bridging the translational gap between rodent and human pain research

Advisor: Robert W. Gereau IV, Ph.D.

Marquette University, Milwaukee, WI (08/2008-05/2012)

B.S., Physiological Sciences, Minor: German

Honors Program, *summa cum laude*

Research Advisor: Edward Blumenthal, Ph.D.

RESEARCH EXPERIENCE

Washington University in St. Louis

St. Louis, MO

Graduate Student with Robert W. Gereau IV, Ph.D.

03/2013-present

- Investigated voluntary behaviors as readouts for rodent models of persistent inflammatory and neuropathic pain
- Evaluated voluntary exercise training as an analgesic intervention in uninjured, inflammatory, and nerve-injured pain states in mice
- Investigated metabotropic glutamate receptors 2/3 as a putative peripheral analgesic targets in mouse and human sensory neurons
- Helped develop culturing methods for human sensory neurons

Rotating Graduate Student with John Cirrito, Ph.D.

11/2012-03/2013

- Examined the role of muscarinic acetylcholine receptor M1 activation in Alzheimer's disease pathology

Rotating Graduate Student with Michael Bruchas, Ph.D.

08/2012-11/2012

- Demonstrated a role for the kappa opioid receptor in the modulation of cold sensation

Marquette University

Milwaukee, WI

Undergraduate Research Assistant/ Summer 2010 Intern with Edward Blumenthal, Ph.D.

08/2008-07/2012

- Discovered that *drop-dead*-mediated cross-linking is required for eggshell integrity in *Drosophila melanogaster*

Max Delbrück Center for Molecular Medicine

Berlin, Germany

DAAD RISE Intern with Enno Klußmann, Ph.D.

06/2011-08/2011

(German Academic Exchange Service Research Internships in Science and Engineering)

- Characterized small molecule inhibitors of aquaporin-2 in mouse medullary collecting duct cells in order to better understand and modulate renal osmotic regulation

RESEARCH TECHNIQUES (• extensive experience; ° some experience)

- Rodent behavioral assays for evaluating pain, motor, and anxiety behavior
- Calcium imaging using chemical calcium indicators
- Culture of primary rodent and human neurons
- Design and implementation of rodent exercise paradigms
- Surgical procedures for rodent models of neuropathic and inflammatory pain
- Biochemical techniques including immunohistochemistry, immunocytochemistry, in situ hybridization, western blotting, and ELISAs
- Software proficiency in Adobe Illustrator and GraphPad Prism
- ° Confocal microscopy
- ° Molecular biology techniques including PCR and RNA isolation
- ° Patch clamp electrophysiology and data analysis using Igor Pro
- ° Stereotaxic surgery and *in vivo* microdialysis

PUBLICATIONS

1. **Sheahan TD**, Valtcheva MV, Pullen MY, McIlvried LA, Baranger DAA, Gereau RW, IV. (In preparation) Metabotropic glutamate receptor 2/3 (mGluR2/3) activation suppresses TRPV1 sensitization in mouse, but not human sensory neurons.
2. Hammarsten CC, Bord A, Shelton ME, Walicki MC, **Sheahan TD**, Golden JP, Gereau RW, IV, McCall JG (In preparation). Unguided behavioral classification using persistent homology.
3. **Sheahan TD**, Siuda ER, Bruchas MR, Shepherd AJ, Mohapatra DP, Gereau RW, IV, Golden JP (2017). Inflammation and nerve injury minimally affect mouse voluntary behaviors proposed as indicators of pain. *Neurobiology of Pain* 2:1-12. doi:10.1016/j.ynpai.2017.09.001.
4. Shepherd AJ, Mickle AD, Copits BA, Karlsson P, Kadunganattil S, Golden JP, Tadinada SM, Mack M, Haroutounian S, de Kloet AD, Samineni VK, Valtcheva MV, McIlvried LA, **Sheahan TD**, Jain S, Ray PR, Usachev YM, Dussor G, Grobe JL, Kim BS, Krause EG, Price TJ, Gereau RW, IV, Mohapatra DP. (Submitted) Angiotensin triggers macrophage-to-sensory neuron crosstalk to elicit neuropathic pain.
5. Valtcheva MV*, Copits BA*, Davidson S, **Sheahan TD**, Pullen MY, McCall JG, Dikranian K, Gereau RW, IV (2016). Surgical extraction of human dorsal root ganglia from organ donors and preparation of primary sensory neuron cultures. *Nature Protocols* 11(10): 1877-1888. (*equal contributions) doi: 10.1038/nprot.2016. PMID: 27606776.
6. **Sheahan TD**, Copits BA, Golden JP, Gereau RW, IV (2015). Voluntary Exercise Training: Analysis of Mice in Uninjured, Inflammatory, and Nerve-Injured Pain States. *PLoS ONE* 10(7): e0133191. doi:10.1371/journal.pone.0133191. PMCID: PMC4510282.
7. **Sheahan TD**, Korthauer LE, Blumenthal EM. (In preparation) The *Drosophila* drop-dead gene is required for eggshell integrity.

PRESENTED ABSTRACTS

1. **Sheahan TD**, Davidson S, Golden JP, Valtcheva MV, Copits BA, Pullen MY, Baranger DAA, McIlvried LA, Ghatti A, Schmidt RE, Ray PR, Price TJ, Gereau RW, IV. mGlu2/3 differentially modulate TRPV1 sensitization in mouse and human sensory neurons. *Journal of Pain, American Pain Society*, 2017.
**Selected as an excellent early career poster.*
2. **Sheahan TD**, Siuda ER, Shepherd AJ, Mohapatra DP, Gereau RW, IV, Golden JG. Voluntary behaviors as readouts for persistent pain in mice. *Society for Neuroscience*, 2016.
3. **Sheahan TD**, Davidson S, Golden JP, Valtcheva MV, Copits BA, Pullen MY, Ghatti A, Schmidt RE, Ray PR, McIlvried LA, Price TJ, Gereau RW, IV. mGlu2/3 suppress sensory neuron sensitization in mouse and human. *Washington University in St. Louis Neuroscience Retreat*, 2016.
4. **Sheahan TD**, Siuda ER, Webb JM, Gereau RW IV, Golden JP. Non-reflexive measures of persistent pain in mice. *Society for Neuroscience*, 2015; *Washington University in St. Louis Graduate Student Research Symposium*, 2016.
5. **Sheahan TD**, Webb JM, Gereau RW, IV, Golden JP. Non-reflexive measures of persistent pain in mice. *Journal of Pain, American Pain Society*, 2015; *Washington University in St. Louis Anesthesiology Academic Evening*, 2015; *Washington University in St. Louis Neuroscience Retreat*, 2015.
6. **Sheahan TD**, Copits BA, Golden JP, Gereau RW, IV. The effects of voluntary exercise on inflammatory and neuropathic pain. *Society for Neuroscience*, 2014.
7. **Sheahan TD**, O'Brien DE, Golden JP, Gereau RW, IV. The effects of voluntary wheel running on pain. *Journal of Pain, American Pain Society*, 2014.
8. **Sheahan TD**, Korthauer LE, Blumenthal EM. Female sterility and compromised eggshell integrity of drop-dead mutants in *Drosophila melanogaster*. *Drosophila Research Conference 2011; Marquette University Summer Research Symposium*, 2010.

ORAL PRESENTATIONS and SEMINARS

"Using human tissue to validate preclinical rodent findings: mGlu2/3 suppress sensory neuron sensitization in mouse and human" (selected abstract). *Department of Anesthesiology Academic Evening*, Washington University in St. Louis School of Medicine, April 2017.

"Bridging the translational gap between rodent and human pain research" (invited speaker). *Pittsburgh Center for Pain Research*, University of Pittsburgh, March 2017.

ACADEMIC and PROFESSIONAL AWARDS

Washington University in St. Louis

Fine Science Tools Travel Fellowship, 2017

Outstanding Abstract, Department of Anesthesiology Academic Evening, 2017

American Pain Society Young Investigator Award 2014, 2015, 2017

Marquette University

Biological Sciences Academic Achievement Award, 2012

Klinger College of Arts and Sciences Gold Medal Award, 2012

Catherine Welsch Smith Research Award, 2011

Honors Program Summer Research Grant, 2011

Barry Goldwater Scholarship Honorable Mention, 2011

TEACHING, OUTREACH, and ACADEMIC SERVICE

Course Master, Molecular Biology at the Cutting Edge	Spring 2017
Lecturer, Molecular Biology at the Cutting Edge <i>"How to Efficiently Read Scientific Papers"</i> <i>"Calcium Imaging of Sensory Neurons: in vitro and in vivo Approaches"</i>	Spring 2016, 2017
Teaching Assistant, Neurophysiology Lab	Fall 2013
Associate, Washington University Center for the Integration of Research, Teaching, and Learning (WU-CIRTL)	2014 - Present
Organizer, Neuroanatomy Outreach at St. Louis University High School <i>Neuroscience lecture and hands-on neuroanatomy for over 250 students</i>	Spring 2016, 2017
Presenter, Neuroanatomy Outreach at St. Louis University High School	Spring 2014-2017
Peer Mentor, BP-ENDURE St. Louis Neuroscience Pipeline	2016
Presenter, St. Louis Science Center Neuroscience Outreach	2013
Ph.D. Candidate Representative, Neuroscience Program Steering Committee	2013-2015

CONTRIBUTIONS to MANUSCRIPT REVIEWS

Neuroscience (1x)	2015
Neuron (1x); Journal of Neuroscience (1x)	2014

PROFESSIONAL SOCIETY MEMBERSHIPS

Society for Neuroscience	2013 - Present
American Pain Society	2014 – 2015, 2017

REFERENCES

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